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4. Title of the invention "NEURODEGENERATIVE DISORDER RELATED GENE"

5. Name of your agent *(if you have one)*

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NEURODEGENERATIVE DISORDER RELATED GENE

The present invention relates to the use of a polynucleotide fragment encoding protein kinase C type I (PKC type I) as well as fragments thereof, mutant polynucleotide fragments of the polynucleotide fragment, a recombinant vector comprising such a polynucleotide fragment or mutant polynucleotide fragment, a host cell comprising said polynucleotide fragment or mutant polynucleotide fragment, a host cell comprising a recombinant vector comprising said polynucleotide fragment or mutant polynucleotide fragment, a recombinant or synthetic polypeptide thereto, antibodies specific to said polypeptide, antisense oligonucleotides complementary to said polynucleotide fragment or mutant polynucleotide fragment, pharmaceutical compositions comprising said recombinant or synthetic polypeptide, pharmaceutical compositions comprising said antisense oligonucleotides and pharmaceutical compositions comprising said polynucleotide fragment for use in prophylaxis and/or as a therapeutic agent in animals, particularly humans, as well as uses of said polynucleotide fragment or mutant polynucleotide fragment, antisense oligonucleotides, antibodies and/or polypeptides in diagnostic and/or screening assays.

Degenerative disorders of the nervous system, such as Parkinson's Disease, Alzheimer's Disease and Huntington's Disease, have provided a challenge for many years, in both the basic research and clinical contexts. A major problem has been the lack of animal models which accurately mimic

the clinical conditions since a large proportion of research is carried out initially on animals in which a disorder has been created by experimental manipulation of the central nervous system (CNS). Examples of experimental manipulation of the CNS in the field of Parkinson's Disease include rodent studies which have relied on lesioning the nigrostriatal system with, for example, the toxin 6-hydroxydopamine. Several genetic mouse models of movement disorders exist, although the majority of such mutants breed poorly and have a reduced life expectancy which limits their efficacy for study of the long term progression of the various conditions. One such model, the weaver mouse, has a deficiency in its dopaminergic systems and has thus been proposed as a model of Parkinson's disease. However, this mutant also possesses severe cerebellar abnormalities and the resulting behaviours may mask those generated by the Parkinsonian-like dopamine deficiencies.

A mutant rat strain (AS/AGU) which had risen spontaneously in a closed breeding colony of Albino-Swiss (AS) rats at the Department of Anatomy, Glasgow University (AGU) was initially described by Clarke & Payne (1994) European Journal of Neuroscience 6 pp885 - 888. The mutant rat displayed a movement disorder which consisted primarily of a difficulty in initiating movement, with a staggering gait and hind limb rigidity. The animals were in good general health and were fertile. Successful breeding between affected individuals resulted after several

generations in all off-spring bearing the same motor deficits. Subsequent genetic analysis has shown that the mutation is an autosomal recessive. The gait disturbances are first detected at around postnatal day 10 and become progressively more severe. The life expectancy of these animals is around 18 months, somewhat foreshortened when compared with the parent Albino-Swiss strain, whose life expectancy is more than 2 years.

A 60% deficit in dopaminergic cell bodies in the substantia nigra pars compacta was detected in the AS/AGU mutants compared to the AS controls at 12 months of age. This provided evidence for basal ganglia involvement and suggested that the disorder could be pathologically very similar to human Parkinson's Disease, which is characterised by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc).

Further research using the micropunch procedure revealed depletions of tissue (combined pre-synaptic and released) dopamine in the dorsal and lateral striatum of 30% and 20% respectively in 12 month old AS/AGU mutants compared to age matched controls (Campbell et al 1996 Neuroscience Letters, 213 pp 173 - 176). This was an expected consequence of loss or decreased function of dopaminergic neurons in the SNpc, which project to the striatum.

When an age range study was carried out on rats of 3 months, 6 months, 9 months and 12 months old, it was found that tissue dopamine depletion in the dorsal and lateral striatum of AS/AGU mutants increased with age from 6 months onwards, thereby demonstrating that the disorder was progressive (Campbell et al, 1997 Neuroscience Letters 239 pp54 - 56).

Extracellular levels of dopamine and its metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC) were measured by microdialysis in the corpus striatum of conscious AS/AGU mutant rats. Extracellular levels of dopamine were found to be very significantly reduced approximately 80% in 9 month old AS/AGU rats compared to age matched AS controls. This was also found to be progressive over an age range. The extracellular levels of the degradation product of dopamine, ie. DOPAC, was found to be elevated in AS/AGU rats compared to AS controls at all ages (Campbell et al, 1998 Neuroscience 85 pp323 - 325).

Local cerebral glucose utilisation is a measure of the metabolic activity of cells in various brain regions. This was measured in AS/AGU rats and statistically significant decreases in glucose utilisation were apparent in 12 out of 44 brain regions examined in 12 month rats. The most significant decreases were found in the substantia nigra pars compacta and the mededical geniculate. Lesser effects were observed in the subthalamic nucleus in extra pyramidal regions and several limbic structures. The cerebellum and white matter areas were not affected. This evidence

suggests that the dopaminergic cells of the SNpc are in some way metabolically comprised (Lam et al, 1998 European Journal of Neuroscience 10 pp1963 - 1967).

When L-Dopa was administered to AS/AGU rats it was shown to greatly enhance the ability of the AS/AGU rats to perform a number of locomotor tasks such as mid-air righting and walking down an inclined ramp. This was also observed when foetal midbrain cells were transplanted into the striatum. L-Dopa treatment and foetal midbrain transplants are known to improve the symptomatic state of human Parkinson's Disease patients. This result revealed that the majority of the movement disorder, and thus the neurodegenerative damage in the AS/AGU is due to loss of dopaminergic neuron function in the SNpc (Payne et al, 1998 Movement Disorders 13 pp832 - 834).

All this work suggested that the AS/AGU rat may be a good candidate as a phenotypic model for Parkinson's Disease. However, there was no evidence of how the AS/AGU rat may be affected at the genetic level.

The PKC gamma gene encoding the protein kinase C type I (PKC type I) isoenzyme has previously been studied in mice and transgenic mice lacking the type I subtype have been produced (Abeliovich et al 1993, Cell, 75, pp1253 - 1262). The null mutant mice produced displayed little or no behavioural impairment.

A mutation in the PKCγ gene in humans has been shown to be associated with the disorder retinitis pigmentosa (RP) see Al-Maghtheh et al, 1998 Am. J. Hum. Genetics 62

pp1248 - 1252. However, there was no suggestion that the mutation was associated with any additional neurological disorder such as Parkinson's Disease, Alzheimer's Disease or Huntington's Disease.

The present invention is based on the discovery by the present inventors that a mutation(s) within the PKCγ gene encoding the type I subtype of protein kinase C is associated with the AS/AGU mutant rat.

Thus, in a first aspect, the present invention provides use of a polynucleotide fragment comprising the PKCγ gene encoding the type I subtype of protein kinase C in the manufacture of a medicament for treating a neurodegenerative disorder.

In a further aspect, the present invention provides use of a polypeptide which comprises protein kinase C type I in the manufacture of a medicament for treating a neurodegenerative disorder.

Typically the medicament may be used to treat mammals, in particular humans. The neurodegenerative disorder may be a degenerative disorder of the central nervous system, such as Alzheimer's Disease, or more particularly, a neurodegenerative disorder associated with dopaminergic cell degeneration and/or movement impairments such as Parkinson's Disease, or Huntington's Disease/Chorea, Dementia with Lewy bodies, Multiple-system atrophy (including striatonigral degeneration, sporadic olivopontocerebellar atrophy and shy-drager syndrome), Progressive supranuclear palsy, cortical-basal ganglionic

(corticobasal) degeneration, vascular Parkinsonism or ballism.

"Polynucleotide fragment" as used herein refers to a polymeric form of nucleotides of any length, both to ribonucleic acid sequences and to deoxyribonucleic acid sequences. In principle, this term refers to the primary structure of the molecule, thus this term includes double stranded and single stranded DNA, as well as double and single stranded RNA, and modifications thereof.

In general, the term "polypeptide" refers to a molecular chain of amino acids with a biological activity. It does not refer to a specific length of the product, and if required it can be modified *in vivo* and/or *in vitro*, for example by glycosylation, myristoylation, amidation, carboxylation or phosphorylation; thus *inter alia* peptides, oligopeptides and proteins are included. The polypeptides disclosed herein may be obtained, for example, by synthetic or recombinant techniques known in the art.

Thus the term extends to cover, for example, polypeptides obtainable from various transcripts and splice variants of these transcripts from the PKCγ gene. Additionally, functional domains may be observed in the protein and isolated polypeptides relating to these functional domains may be of particular use. For example, a regulatory domain, a kinase domain and an ATP-binding domain have been observed in the PKC type I polypeptide. The present invention also relates to polynucleotide fragments comprising a nucleotide sequence encoding such

functional domain polypeptides.

It will be understood that for the PKCγ nucleotide and polypeptide sequences referred to herein, natural variations can exist between individuals. These variations may be demonstrated by amino acid differences in the overall sequence or by deletions, substitutions, insertions or inversions of amino acids in said sequence. All such variations are included in the scope of the present invention.

As is well known in the art, the degeneracy of the genetic code permits substitution of bases in a codon resulting in a different codon encoding the same amino acid. Consequently, it is clear that any such derivative nucleotide sequence based on the sequences disclosed herein is also included in the scope of the present invention.

Thus, the present invention also includes nucleotide sequences similar to the polynucleotide sequences disclosed herein. It is understood that similar sequences include sequences which remain hybridised to the polynucleotide sequences of the present invention under stringent conditions. Typically, a similar test sequence and a polynucleotide sequence of the present invention are allowed to hybridise for a specified period of time generally at a temperature of between 50 and 70°C in double strength SSC (2 x NaCl 17.5g/l and sodium citrate (SC) at 8.8 g/l) buffered saline containing 0.1% sodium dodecyl sulphate (SDS) followed by rinsing of the support at the same temperature but with a buffer having a reduced SSC

concentration. Depending upon the degree of similarity of the sequences, such reduced concentration buffers are typically single strength SSC containing 0.1% SDS, half strength SSC containing 0.1% SDS and one tenth strength SSC containing 0.1% SDS. Sequences having the greatest degree of similarity are those the hybridisation of which is least affected by washing in buffers of reduced concentration. It is most preferred that the similar and inventive sequences are so similar that the hybridisation between them is substantially unaffected by washing or incubation in one tenth strength SSC containing 0.1% SDS.

Furthermore, fragments derived from the PKCγ gene or PKC type I protein which still display PKCγ specific properties or PKC type I specific properties are also included in the present invention. "PKCγ specific properties" is understood to relate to biological functions which are attributable to naturally-occurring PKCγ gene and "PKC type I specific properties" is understood to relate to biological functions which are attributable to naturally-occurring PKC type I protein. This may include fusion proteins.

All such modifications mentioned above resulting in such derivatives of PKCγ are covered by the present invention so long as the characteristic PKCγ properties remain substantially unaffected in essence.

The present inventors applied genetic mapping techniques in order to ascertain the genotypic variation displayed in the AS/AGU rat using the process of "positional cloning"

(Collins, 1992, Nature Genetics, 1, 3 - 6). This mapping revealed that the AS/AGU mutation was in close proximity to the genetic marker R158 (Serikawa et al, 1992, Genetics 137, pp701 - 721). Nucleotide sequencing was then carried out, which upon comparison with wild type AS sequence, revealed a mutation in the PKCγ gene.

A point mutation was observed at nucleotide 841 of the rat PKCγ messenger RNA sequence(shown in Figure 1 where nucleotide numbering is such that base A of the ATG start codon is no. 1) such that a guanine base, present in the AS gene sequence, was mutated to a thymine base in the AS/AGU mutant sequence. This transversion mutation results in the generation of an in-frame stop codon which upon translation of the PKCγ gene would result in a prematurely terminated protein the length of which would be 280 amino acids. It is postulated that this truncated protein would not possess several of the domains present in the wild-type protein. That is, the regulatory domain would be present in the truncated protein and not the kinase or ATP-binding domains.

Since this genotypic mutation is associated with the phenotypic movement disorder observed in the AS/AGU mutant rat, observation of such a mutation in PKCγ may be used in a predictive test for neurodegenerative disorders, such as Parkinson's disease, Alzheimer's Disease or Huntington's Disease. Alternatively, measuring levels of PKC type I protein levels and/or activity may be useful in a predictive or diagnostic test for neurodegenerative

disorders.

Thus, in a further aspect, the present invention provides a method of testing an animal thought to have or predisposed to having a neurodegenerative disorder which comprises detecting the presence of a mutation in the PKCγ gene and/or its associated promoter.

Typically, the mutation(s) may result in a truncated product from the PKCγ gene being produced. More particularly the mutation may occur in the 5' half of the gene. For example the mutation may be a point mutation such as at position 841 of the rat PKCγ gene or similar region of the PKCγ gene from another species. The skilled man will immediately appreciate that the information presented herein relating to the rat PKCγ may easily be equated or correlated with a similar mutation at a corresponding location in the PKCγ gene from another species, such as humans. Thus, the present invention provides the means with which to test humans for a similar mutation in the human PKCγ gene and therefore predict if the test subject has or is predisposed to developing a neurodegenerative disorder for example, Parkinson's Disease, Alzheimer's Disease or Huntington's Disease.

Typical techniques for detecting the mutation may include restriction fragment length polymorphism, hybridisation techniques, DNA sequencing, exonuclease resistance, microsequencing, solid phase extension using ddNTPs, extension in solution using ddNTPs, oligonucleotide ligation assays, methods for detecting single nucleotide

polymorphisms such as dynamic allele-specific hybridisation, ligation chain reaction, mini-sequencing, DNA "chips", allele-specific oligonucleotide hybridisation with single -or dual-labelled probes merged with PCR or with molecular beacons, and others.

Increased levels of the mRNA transcripts encoding the truncated PKC type I polypeptide have been observed. This may be due to upregulation in the synthesis of the mRNA derived from the mutant PKCγ gene in an attempt to produce functional PKC type I protein. Thus, detection of increased levels of the truncated mRNA transcript or mRNA precursors, such as nascent RNA, may be used to diagnose if the test subject has or is predisposed to developing a neurodegenerative disorder.

The information presented herein may also be used to genetically manipulate the wild-type PKCγ gene, mutant PKCγ gene or derivatives thereof, for example to clone the gene by recombinant DNA techniques generally known in the art. Cloning of homologous genes from other species of mammal may be performed with this information by widely known techniques; for example, suitable primers may be designed to a consensus region and/or functional domains of the sequence shown in Figure 2 and such primers used as probes for cloning homologous genes from other organisms.

Moreover, mammalian PKCγ mutant and wild-type nucleotide sequences of the present invention are preferably linked to expression control sequences. Such control sequences may comprise promoters, operators,

inducers, ribosome binding sites etc. Suitable control sequences for a given host may be selected by those of ordinary skill in the art.

A nucleotide sequence according to the present invention can be ligated to various expression-controlling DNA sequences, resulting in a so-called recombinant nucleic acid molecule. Thus, the present invention also includes an expression vector comprising an expressible PKCγ mutant or wild-type nucleotide sequence. Said recombinant nucleic acid molecule can then be used for transformation of a suitable host.

Such recombinant nucleic acid molecules are preferably derived from for example, plasmids, or from nucleic acid sequences present in bacteriophages or viruses and are termed vector molecules.

Specific vectors which can be used to clone nucleotide sequences according to the invention are known in the art (eg. Rodriguez and Denhardt, editors, Vectors: A survey of molecular cloning vectors and their uses, Butterworths, 1988).

The methods to be used for the construction of a recombinant nucleic acid molecule according to the invention are known to those of ordinary skill in the art and are *inter alia* set forth in Sambrook et al, Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory, 1989.

The present invention also relates to a transformed cell comprising the mutant or wild-type nucleic acid molecule in an expressible form. "Transformation", as used herein, refers to the introduction of a heterologous nucleic acid sequence into a host cell *in vivo*, *ex vivo* or *in vitro* irrespective of the method used, for example, by calcium phosphate co-precipitation, direct uptake or transduction.

The heterologous nucleic acid sequence may be maintained through autonomous replication or alternatively may be integrated into the host's genome. The recombinant DNA molecules are preferably provided with appropriate control sequences compatible with the designated host which can regulate the expression of the inserted nucleic acid sequence.

The most widely used hosts for expression of recombinant nucleic acid molecules are bacteria, yeast, insect cells and mammalian cells. Each system has advantages and disadvantages in terms of the vector used, potential ease of production and purification of a recombinant polypeptide and authenticity of product in terms of tertiary structure, glycosylation state, biological activity and stability and will be a matter of choice for the skilled addressee.

In addition to promoting expression of a PKC type I polypeptide in cells, in certain circumstances it may be advantageous to substantially prevent or reduce the expression or activity of the native PKC type I in a host,

for example, for the production of animal models for use in drug screening, or particularly if the native PKC type I is of a mutant form.

Thus, according to a further aspect of the invention, there is provided an antisense nucleotide fragment complementary to a PKCγ nucleotide sequence of the present invention. Included in the scope of "antisense nucleotide fragment" is the use of synthetic oligonucleotide sequences, or of equivalent chemical entities known to those skilled in the art, for example, peptide nucleic acids. Further, such sequences can be used as part of ribozyme and/or triple helix sequences, which may also be useful for target gene regulation. Also provided is a nucleotide fragment comprising a nucleotide sequence which, when transcribed by the cell, produces such an antisense fragment. Typically, antisense RNA fragments will be provided which bind to complementary PKCγ mRNA fragments to form RNA double helices, allowing RNAse H to cleave the molecule and rendering it incapable of being translated by the cell into polypeptides.

A further aspect of the present invention provides antibodies specific to the PKC type I polypeptide or truncated polypeptide as identified herein or epitopes thereof. Production and purification of antibodies specific to an antigen is a matter of ordinary skill, and the methods to be used are clear to those skilled in the art. The term antibodies can include, but is not limited to, polyclonal antibodies, monoclonal antibodies (mAbs),

humanised or chimeric antibodies, single chain antibodies, Fab fragments, $F(ab')_2$ fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope binding fragments of any of the above. Such antibodies may be used in modulating the expression or activity of the full length or truncated PKC type I polypeptide, or in detecting said polypeptide *in vivo* or *in vitro*.

The present invention further provides a recombinant or synthetic PKC type I polypeptide for the manufacture of reagents for use as prophylactic or therapeutic agents in mammals. In particular, the invention provides pharmaceutical compositions comprising the recombinant or synthetic PKC type I polypeptide together with a pharmaceutically acceptable carrier therefor.

According to a still further aspect of the present invention, there is provided use of a polypeptide or nucleic acid sequence as hereinbefore described for promoting nervous system degeneration for use in, for example, production of animal models which may be used in drug screening.

There is also provided use of a polypeptide or nucleic acid sequence as hereinbefore described in preventing, delaying, treating or inhibiting degeneration of the nervous system. There is further provided a method of preventing, delaying, treating or inhibiting degeneration of the nervous system comprising providing PKC type I polypeptide to a subject displaying or predicted to display

degeneration of the nervous system. Such a method may find particular application in the treatment of degenerative disorders of the central nervous system, such as Alzheimer's Disease, or more particularly neurodegenerative disorders associated with movement impairment such as Parkinson's Disease or Huntington's Chorea.

Also provided is a method of preventing, delaying, treating or inhibiting degeneration of the nervous system comprising providing a subject with a nucleotide sequence or an antibody which substantially prevents or reduces expression or activity of a mutant PKC type I polypeptide.

A yet further aspect of the present invention provides use of polypeptides or nucleic acid sequences as hereinbefore described in the treatment of degenerative disorders of the nervous system, such as Parkinson's Disease, Alzheimer's Disease or Huntington's Disease. One such envisaged treatment may be by way of so-called gene therapy in which a wild-type PKCγ gene is introduced to a subject possessing a mutant PKCγ gene in order to counter the effects of the mutant PKCγ gene. This may be performed by the implantation of cells, such as fibroblasts expressing human or mammalian PKCγ fused to herpes virus VP22 protein that will transfer itself and PKCγ into adjacent neurons. Transformation of the cells to be implanted may be performed *in vitro* by any number of techniques, including physical means such as microinjection, electroporation, bioballistic or particle bombardment, jet injection or others; by chemical means

such as using calcium phosphate, DEAE dextran, polylysine conjugates, "starburst" dendrimer conjugates, polybrene-dimethyl sulphoxide. The PKCγ gene itself, within an appropriate vector end-linked to an appropriate expression system, may be directly delivered via receptor-mediated uptake systems such as asialoglycoprotein and transferrin, liposomes, virus-like particles, intracellular targeting ligands and others; and by biological means including retroviral vectors such as Moloney murine leukaemia virus, adenovirus vectors and adeno-associated virus vectors, Herpes Simplex virus vectors, Semliki Forest virus vectors, Sindbis virus vectors and others.

The present invention also relates to methods for prognostic and diagnostic evaluation of various degenerative disorders of the nervous system, and for the identification of subjects who are predisposed to such disorders, for example determination of allelic variation by determination of the PKCγ nucleotide sequence in an individual and/or detection of truncated transcripts derived from the PKCγ gene, whether they are mRNA or polypeptide or measurements of PKC type I levels and/or activity. Furthermore, the invention provides methods for evaluating the efficacy of drugs for such disorders and monitoring the progress of patients involved in clinical trials for the treatment of such disorders.

The invention further provides methods for the identification of compounds which modulate the expression of a mutated or wild-type PKCγ gene and/or the activity of

the product(s) of such a mutant or wild-type PKCγ gene which may be involved in processes relevant to degenerative disorders of the nervous system. Such compounds may include agonists, defined as compounds which increase the expression of a mutated or wild-type PKCγ gene and/or activity of the product(s) of such a mutant or wild-type PKCγ gene, and/or antagonists, defined as compounds which decrease the expression of a mutated or wild-type PKCγ gene and/or the activity of the product(s) of such a mutant or wild-type PKCγ gene. Thus, the present invention in a further aspect also provides agonists and/or antagonists.

The biological function of the PKCγ gene can be more directly assessed by utilizing relevant *in vivo* and *in vitro* systems. *In vivo* systems can include, but are not limited to, animal systems which naturally exhibit the symptoms of nervous system disorders, or ones which have been engineered to exhibit such symptoms. Further, such systems can include, but are not limited to, transgenic animal systems. *In vitro* systems can include, but are not limited to, cell-based systems comprising PKCγ gene/PKC type I protein expressing cell types. The cells can be wild type cells, or can be non-wild type cells containing modifications known or suspected of contributing to the disorder of interest.

In further characterising the biological function of the PKCγ mutant or wild-type gene, the expression of the PKCγ mutant or wild-type gene can be modulated within the *in vivo* and/or *in vitro* systems, i.e. either overexpressed

or underexpressed in, for example, transgenic animals and/or cell lines, and its subsequent effect on the system can then be assayed. Alternatively, the activity of the product of the identified gene can be modulated by either increasing or decreasing the level of activity in the *in vivo* and/or *in vitro* system of interest, and its subsequent effect then assayed.

The information obtained through such characterisations can suggest relevant methods for the treatment or control of nervous system disorders. For example, relevant treatment can include a modulation of gene expression and/or gene product activity. Characterisation procedures such as those described herein can indicate whether such modulation should be positive or negative. As used herein, "positive modulation" refers to an increase in gene expression or activity of the gene or gene product of interest. "Negative modulation", as used herein, refers to a decrease in gene expression or activity.

In vitro systems can be designed to identify compounds capable of binding the PKCγ mutant or wild-type gene products of the invention. Compounds identified, for example, could be useful in modulating the activity of wild type or mutant PKCγ gene products, could be useful in elaborating the biological function of the PKCγ gene products, or could disrupt or enhance normal PKCγ gene product interactions, for example, the activators or inhibitors of PKC type I protein as disclosed in Keenan et al, 1997, FEBS Letters, 415 pp101 - 108. Such compounds

may be investigated for their use in treating or alleviating motor impairment and/or dopaminergic cell degeneration disorders.

These and other aspects of the invention shall now be further described, by way of example only, and with reference to the accompany figures which show:

Figure 1 is a diagram illustrating the region of rat genome selected to contain recombination events and genetic markers used to establish panels of backcross progeny recombinant in the interval containing the mutant PKCγ gene hereinafter referred to as nng3. The three backcrosses (BN x NNG3) F1 x NNG3, (F344 x NNG3) F1 x NNG3 and (DA x NNG3) F1 x NNG3 are labelled BN, F344 and DA respectively;

Figure 2 is a sequence alignment of sequence obtained from the PKCγ gene in rat strains NNG3 and AS. Sequences are aligned to the rat (Rattus rattus) mRNA sequence obtained from NCBI (Accession number: X07287). The point mutation identified within the nng3 sequence is shown in bold and underlined at nucleotide 841. The translation start site is shown underlined, and the microsatellite defining the marker R158 (within the 3'UTR) is shown in italics. The primers defining the marker R158 are shown underlined and indicated by arrows. The normal translation stop site is shown at nucleotide 2093, in bold and underlined;

Figure 3 is an alignment of PKCγ DNA sequence as illustrated in Figure 2 with the predicted amino acid sequence. The sequence illustrated is from the rat strain *Rattus rattus*, and the nucleotide conversion seen in the sequence illustrated in Figure 2 is given in bold, below the sequence for amino acid number 281. The normal translational start and stop codons are shown in bold. The site at which a codon encoding the amino acid Glu is changed by the mutation to a STOP codon, resulting in a polypeptide terminator, is also indicated and is shown in bold;

Figures 4 (a) and (b) are immunocytochemistry stainings of rat brains with anti-PKCγ antibody. Figure 4(a) illustrates AS control rat brain stained with PKCγ, with the Purkinje cell layers 10 and granule cell layers 15 indicated. Figure 4(b) illustrates NNG3 rat brain stained with anti-PKCγ antibody;

Figure 5 illustrates a western blot of total brain proteins from the NNG3 and AS strains probed with an anti-PKCγ antibody. The lane marker (M) contains a BENCHMARK™ prestained protein ladder (GibcoBRL) and the sizes of the bands are indicated on the left-hand side. The lanes marked AS and NNG3 contain proteins isolated from brain tissue from AS and NNG3 rat strains, respectively; and

Figure 6 illustrates an in situ hybridisation examination of the PKCγ mRNA transcript in the rat strains NNG3 and AS, as labelled. The brain sections from each strain were consecutive and all sections were probed simultaneously.

Experiment 1 - Genetic fine mapping of the nng3 mutation by genotyping of backcross progeny with R158

The present inventors applied genetic mapping techniques in order to ascertain the genotypic variation displayed in the AS/AGU strain using the process of "positional cloning" (Collins, 1992, Nature Genetics, 1, 3 - 6). Application of this approach relied upon initially determining the chromosomal localisation of the gene by demonstrating linkage to known marker genes. This was followed by additional fine mapping to narrow down the genetic region containing the gene, followed by either sequencing of the region or selection of mRNA transcripts from the region.

Genetic linkage is a direct consequence of the physical linkage of two or more genes with the same pair of DNA molecules that define a particular set of chromosome homologs within the diploid genome (Silver, 1995, Mouse Genetics: Concepts and Applications, Oxford University Press). Generally, crossing over occurs at random sites along all the chromosomes in the mammalian genome. A direct consequence of this randomness is that the further apart two linked loci are from each other, the more likely it is that a crossover event will occur some where within

the length of chromosome which lies between them (Silver, 1995). Thus, the frequency of recombination provides a relative estimate of the genetic distance between a known marker gene and a previously unknown gene.

Three backcrosses were established with the NNG3 strain and strains F344, BN and DA (Festing, 1979 Inbred Strains in Biomedical Research, London : The MacMillan Press Ltd.) to allow mapping of the nng3 gene. The F344, BN and DA rat strains were chosen as they exhibited the highest variation within microsatellite sequences when compared with the NNG3 strain (Shiels et al, 1995, Mammalian Genome, 6, 214 - 215). Microsatellite sequences are defined as tandem repeats of simple dinucleotide or other DNA sequences which occur in allelic forms of various lengths. They are considered convenient genetic markers and are examined by assessing the length of a short polymerase chain reaction product containing the microsatellite using gel electrophoresis.

To establish a backcross, each strain in question (DA, F344 and BN) was crossed to the NNG3 strain and the resulting heterozygous F1 progeny were backcrossed to the NNG3 strain. The resulting backcross progeny were then genotyped to identify if a cross-over event had occurred between the gene of interest and any genetic marker. This allowed positioning of the gene to within a particular chromosome or sub-chromosomal region.

In total 3188 backcross progeny were produced from the three backcrosses. A whole genome scan was carried out with 73 microsatellite markers involving genotyping at least one informative marker per rat chromosome. All of these markers were assayed for linkage to the nng3 mutation. Genetic linkage was observed with the marker R33 (Serikawa et al, 1992, Genetics, 131, 701 - 721), which was localised to chromosome 1 and mapped approximately 30 cM from the nng3 gene. A range of markers from within this chromosomal region were then used to genotype all of the backcross progeny. The closest marker loci bracketing the nng3 mutation were chosen to establish a chromosomal interval within which to carry out precise mapping. This interval was different for each backcross, as shown in Figure 1. This allowed fine mapping of the region and identified recombination events between markers. The markers used are shown in Figure 1 for the three backcrosses (BN X NNG3) F1 X NNG3,(F344 X NNG3) F1 X NNG3 and (DA X NNG3) F1 X NNG3.

All progeny identified to have recombination events within the interval under investigation were genotyped using the genetic marker R158 (Serikawa et al, 1992). The genetic marker R158 consists of a pair of PCR primers which amplify a $(CA)_{25}$ microsatellite repeat from the 3'UTR of the PKCγ gene. The three rat strains BN, F344 and DA were shown to be informative for R158, that is, the length of the microsatellite was shown to vary between strains, when compared to the NNG3 rat strain. The genotyping was

carried out by PCR, as described in the following experimental section, on genomic DNA. The PCR products were then resolved by gel electrophoresis either on 6% acrylamide or on 4% metaphor (Flowgen) agarose gels.

From this experiment (n=3188), no animals were observed to contain a recombination event between the nng3 mutation and the marker R158. This positioned the nng3 mutation $0 \pm < 0.06$ cM from the genetic marker R158. In the mouse, 0.06 cM corresponds to approximately 60 kb of chromosome DNA length. These mapping experiments show that the nng3 mutation (and gene) is very close to the R158 microsatellite, which is itself within the gene PKCγ encoding the type I isoform of protein kinase C. Thus, the nng3 gene is very likely to be PKCγ itself or an immediately adjacent gene.

<u>Experiment 2 - Demonstration of a DNA sequence difference between the allelic forms of the PKCγ gene in the strain and the parent AS strain</u>

The genetic mapping evidence implicated the PKCγ gene as the location of the nng3 mutation. If this implication was correct, a DNA sequence difference must have existed between the allelic forms of the PKCγ gene in the NNG3 strain and in the AS strain from which the NNG3 strain arose by spontaneous mutation. Thus, the following experiment was performed to provide evidence of the sequence difference.

RNA was isolated from 12 month old rats from both the mutant (NNG3) and control (AS) strains. RNA was isolated using TRI REAGENT™ (Sigma) as per the protocol supplied by the manufacturer. RNA was isolated from 1g of brain tissue and homogenised in 10 ml of TRI REAGENT™. 1μg of RNA was then used to synthesise cDNA using the Oligo $(dT)_{12-18}$ Superscript™ Preamplification System for First Strand cDNA Synthesis kit from Gibco BRL. 50ng of cDNA was then used as template in a PCR reaction. The PCR reaction was carried out using 1 unit of *Taq* DNA polymerase (Promega) in a reaction containing 1x magnesium free Thermo buffer, 1mM magnesium chloride (all supplied with *Taq* polymerase from Promega), 125μM dNTPs (Promega) and forward and reverse primers at 5ng/μl each. A hot start was always performed in the PCR reaction. The sequences of all PCR primers used are given in Table 1, along with the PCR reactions in which they were used.

Primer name	Primer sequence 5' to 3'	Utility
Neup113 Forward	GCTACTCAAGGCTCCTGTGGATGG	RT-PCR
Neup114 Reverse	ATGAGATTACATGACGGGCACA	RT-PCR
Neup120 Forward	CAAGGCTCCTGTGGATGGATGG	Genomic PCR
Neup120 Reverse	GCTGCAGTTGTCAGCATCGGC	Genomic PCR

Table 1 - PCR primers utilised. Sequences of PCR primers used are given along with the reactions in which they were used.

The PCR parameters used for *Taq* DNA polymerase were as follows:

113Forward/114Reverse	120 Forward + Reverse
99°C - 3 mins	99°C - 10 mins
80°C - Taq added	80°C - Taq added
Followed by 35 cycles of:	Followed by 30 cycles of:
94°C - 15 secs	94°C - 15 secs
55°C - 30 secs	55°C - 30 secs
72°C - 1 min. Followed by:	72°C - 30 secs. Followed by:
72°C for 10 mins.	72°C - 10 mins.

PCR products were then resolved on a 2% agarose gel (Boehringer Mannheim) and then extracted from the gel using the Qiaquick™ gel extraction kit prior to sequencing.

Sequencing of the PCR products was carried out on the ABI 373 stretch automatic sequencer using the Big Dye terminator chemistry (Perkin Elmer). 3.2 pmoles of a primer used in the PCR reaction were used for sequencing.

PCR reactions were also carried out on rat genomic DNA isolated from rat spleen taken from both rat strains. DNA was isolated using the Pure Gene DNA Isolation kit (Gentra). PCR reactions were carried out as before containing 100 ng of genomic DNA as template. The initial denaturation step of the PCR reaction was also increased to 10 minutes.

PCR reactions were also carried out using proof-reading DNA polymerase enzymes to eradicate errors during DNA polymerisation. The enzymes and modifications of the PCR reactions were as follows: *Tli* polymerase (Promega) reactions were carried out with 1.25 units of the enzyme,

which extends at 74°C. The High Fidelity (HF) kit from Clontech has an automatic hot start and anneals and extends concurrently at 68°C for 3 minutes.

Three different populations of cDNA were synthesised and PCR products obtained from these sequences (a summary of experimental results is given in Table 2). All PCR products sequenced exhibited a G nucleotide in the AS rat strain at position 841 and a T nucleotide at the same position in the NNG3 rat strain, as shown in Figure 2. This transversion mutation creates a new termination or stop codon for translation of PKCγ, resulting in a prematurely terminated protein (Figure 3). As the kinase active domain of the protein is lost, this will result in a loss of ability to phosphorylate the target protein substrates, leading to a cascade of biological consequences. This proposed loss of function appears to be in agreement with the recessive nature of the mutation.

Experiment no.	Template	PCR Enzyme	Summary
1	cDNA: 1	Taq	G at position 841 in AS and T at 841 in NNG3
2 A: B:	 cDNA: 2 cDNA: 1	 Taq Taq	G at position 841 in AS and T at 841 in NNG3
3	cDNA: 2	Tli (proof-reading)	G at position 841 in AS and T at 841 in NNG3
4	cDNA: 2	HF-kit (proof-reading)	G at position 841 in AS and T at 841 in NNG3
5	cDNA: 3	HF-kit	G at position 841 in AS and T at 841 in NNG3
6	Genomic DNA	Taq	G at position 841 in AS and T at 841 in NNG3
7	Genomic DNA	Tli	G at position 841 in AS and T at 841 in NNG3

Table 2 - Summary of sequencing experiments carried out. The table details the enzymes and templates used in PCR reactions carried out for sequencing. The nucleotide number referred to in the table (841) is taken from Figure 2.

These mapping and sequencing results demonstrate that a sequence difference does appear to occur between the NNG3 and AS strains. It is conceivable that this base change (G to T at position 841, Figure 2) in the NNG3 strain gives rise to the phenotype observed in this strain.

Experiment 3 - Detection of PKC gamma by immunocytochemistry

It was postulated that the stop codon in the PKCγ gene in the NNG3 strain led to premature termination of the PKC type I polypeptide. This would result in the protein having no kinase activity. The binding of antibodies raised against the carboxy terminal portion of the PKC type I protein (Boehringer Mannheim) to brains isolated from the NNG3 strain were thus investigated.

PKC gamma was detected by immunocytochemistry using standard protocols with the following modifications: rat brains were taken from 9 months old male rats and were fixed overnight in 4% paraformaldehyde. Brains were then trimmed, placed on a block and sections cut at 50 microns using a vibratome. Paired sections were placed into blocking serum (10% NGS - normal goat serum) and placed on shaker for 1 hour at room temperature. Sections were then placed into primary antibody (rabbit anti-peptide antibody generated using a synthetic peptide corresponding to amino acids 306 - 318 of rat PKCγ, 1:100 - 1:2000, Boehringer Mannheim) overnight at 4°C. Slides were then washed three times in PBS for 5 mins each. Sections were then placed into secondary antibody (biotinylated sheep anti-rabbit IgG antibody, Vector) for 1 hour. Sections were again washed three times in PBS (5 mins each). Vecta stain ABC complex (Vector) was then added to the sections for 1 hour and placed on a shaker. Sections were again washed three times in PBS (5 mins each) and then washed once in PB for 5 mins.

3,3'Diaminobenzidine tetrahydrochloride (DAB) was applied to the sections for 5-10 mins. Sections were washed twice in PB (5 mins each) and the sections finally dehydrated, cleared and mounted.

Figures 4(a) and (b) illustrate the difference between the NNG3 strain and the AS parent strain, from which NNG3 is derived.

A very striking depletion of PKCγ positive cells in Purkinje cells of the NNG3 strain was observed when compared to an age matched AS control. The Purkinje cell layer is a region where PKCγ is predominantly expressed when compared to other PKC isoforms.

These results are consistent with a loss of the carboxy-terminal part or all of the PKC type I protein.

<u>Experiment 4 - Western blot detection of Type I PKC in rat brain protein extracts by antibody hybridisation</u>

A Western blot experiment was carried out to determine the level of expression of the Type I PKC protein in brains from the rat strains AS and NNG3. Specifically, antibodies raised against a peptide located in the carboxy terminal to the truncation site in the PKCγ type I protein of the NNG3 strain were used in order to confirm the lack of expression of this region of the protein.

Total brain proteins were extracted from male, 9 month old rats from both the AS and NNG3 stains. The proteins were isolated from 0.2g of brain tissue using TRI-REAGENT™ (Sigma) and suspended in 2% SDS. 50μg of total proteins

were resolved on a 10% SDS-PAGE (polyacrylamide gel electrophoresis) gel at 200V for 45 minutes. The proteins were then transferred to nitrocellulose (Amersham Life Science) in a wet Western blotter at 30V overnight. The nitrocellulose was then blocked for 1 hour at room temperature, in a solution of Tris-buffered saline (20 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 0.05% Tween 20 (TBST) and 1% bovine serum albumin (BSA). The nitrocellulose was then incubated in TBST plus 1% BSA containing 2 μg/ml anti-PKCγ (Rabbit anti-peptide antibody generated using a synthetic peptide corresponding to amino acids 306-318 of PKCγ, GibcoBRL). The blot was then washed 3 times in TBST for 10 minutes each. The blot was incubated for one hour at room temperature in TBST containing anti-rabbit Ig, peroxidase-linked species-specific whole antibody (from donkey, Amersham Life Science) at a dilution of 1/1000. The blot was then washed as detailed above. After washing the blot was incubated with ECL Western blotting detection reagents (Amersham Pharmacia biotech) and then exposed to autoradiography film (Fuji XR).

This experiment was performed on proteins extracted from the NNG3 strain and the control rat strain (AS). The results obtained are illustrated in Figure 5. A band of the predicted size (80 kDa) was obtained from the AS strain protein extract, whereas no signal was obtained in the NNG3 strain protein extract. The anti-PKCγ antibody recognises an epitope corresponding to amino acids 306-318 of the

protein. In NNG3 it is postulated that a truncated protein is produced which terminates at amino acid 281; therefore, these results illustrate that the epitope for antibody binding is not present in the NNG3 protein, as predicted.

Experiment 5 - *In situ* hybridisation investigation of the PKCγ mRNA transcript in AS and NNG3 rats

An *in situ* hybridisation experiment was carried out to determine the level of expression of the mRNA encoding Type I PKC in the rat strains AS and NNG3.

An antisense oligonucleotide probe was designed to the 3' region of the PKCγ MRNA (nucleotides 2085-2326, numbers taken from Figure 2) and was synthesised and purified by HPLC (GibcoBRL). The sequence of the oligonucleotide was as follows:

5' GCA CTG GGA ACA CCT AGC GGC AGC AGA TGA GAT TAC ATG ACG 3'

Whole brains were taken from 8 month old AS and NNG3 rats. These brains were mounted and sectioned horizontally in 13 micron sections and the sections thaw-mounted onto poly-L-lysine treated microscope slides. The sections were then fixed in 4% paraformaldehyde in 1x PBS (phosphate buffered saline, all solutions were made with (diethylpyrocarbonate-treated water) on ice for 5 minutes, followed by PBS for 2 minutes, then dehydrated in 70% ethanol for 2 minutes, 95% ethanol for 5 minutes and stored in 100% ethanol at 4°C until required.

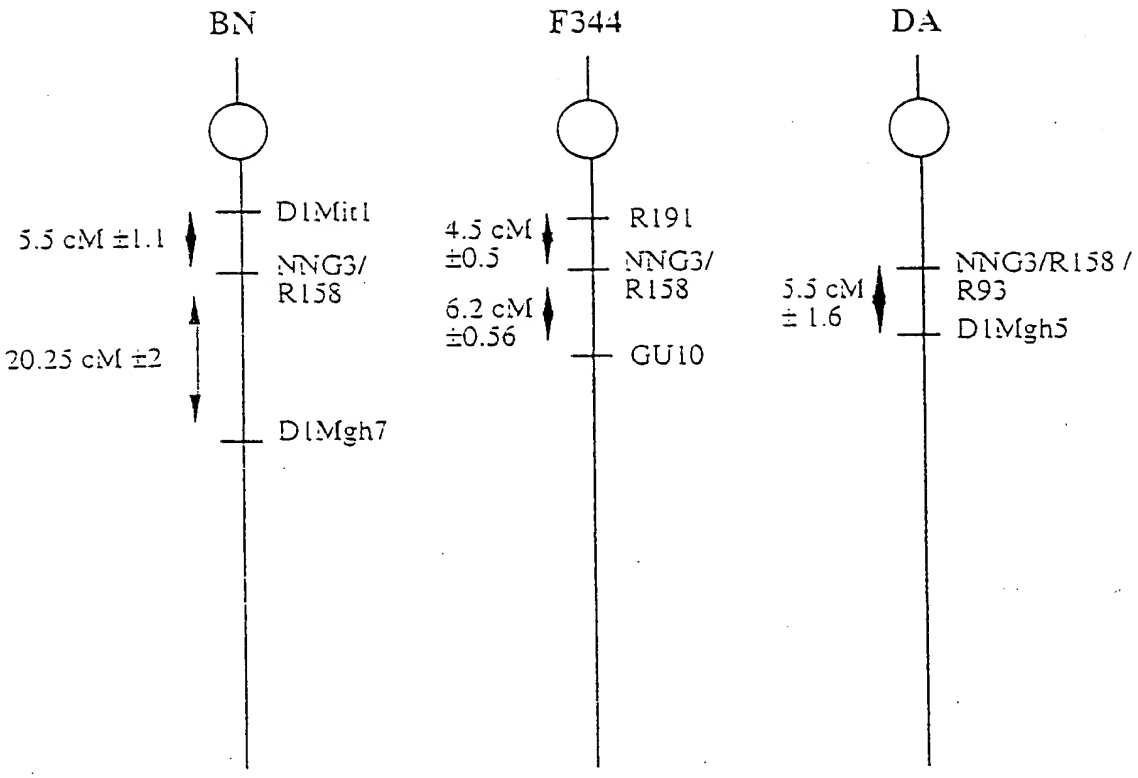
The *in situ* hybridisation probes are labelled and prepared for hybridisation in the following way:

40ng of the oligonucleotide was labelled in a reaction volume of 12.5μl made up in DEPC-water containing 1x reaction buffer (supplied with enzyme), 25μCi of S^{35} α-dATP and 36 units of Terminal deoxynucleotidyl transferase (TdT, FPLC pure, Pharmacia). The reaction was then incubated at 37°C for 1 hour. Purification columns were constructed in 1 ml plastic syringes with GF/C filter paper, cut with a No. 2 cork borer, placed at the bottom of the syringe and the syringe packed with G50 Sephadex (Pharmacia). The column was then centrifuged at 2,000 rpm for 1 min to pack the Sephadex. 87.5 μl of DEPC-treated water was then added to the probe to make to 100 μl. The probe was added to the column and centrifuged for a further 1 minute at 2,000 rpm in a 1.5 ml centrifuge tube for collection. The eluant was then collected and the volume measured. The specific activity of the probe was determined by placing 2 μl of the eluted probe in a scintillation vial and adding 5 ml of scintillant. The specific activity of the probe was then measured in a scintillation counter uwing the Tritium channel. The probe was then standardised so as produce 2 x 10^3 disintegration's per minute/ml of hybridisation mix 50% formamide, 4 x SSC, 10% dextran sulphate, 5 x Denardt's, 200μg/ml acid-alkali cleared salmon sperm DNA, 100μg/ml long chain polyadenylic acid, 120μg/ml heparin, 25mm sodium phosphate, pH7, 1mm pyrophosphate, then DDT added to a final concentration of 20mm. A 100 times excess

of cold oligonucleotide was also added to the control hybridisation mix.

Serial sections were selected for hybridisation so as to be pair-matched and spread throughout the brain. The sections were covered with 200 µl of the appropriate hybridisation mix and then covered with a parafilm coverslip. The slides were then incubated overnight at 42°C. The following day the coverslips were floated off in 1 x SSC at room temperature and the sections were washed twice in a shaking water-bath at 55°C in 1x SSC containing 4 mM DTT for 30 minutes. The sections were then dehydrated through 1x SSC for 30 seconds, 0.1x SSC for 45 seconds, then 70% ethanol for 2 minutes and finally 100% ethanol for 5 minutes. The slides were then allowed to air dry. Once dry the slides were taped to 3MM filter paper and exposed to autoradiography film (Kodak Bio-max MR film, single coated) at room temperature for 1 week.

The results of the *in situ* hybridisation experiment are illustrated in Figure 6. It is evident that the level of PKCγ mRNA in the NNG3 strain appears to be up-regulated when compared to age-matched control brains from the AS strain. This could be explained by some kind of feed-back control system which is attempting to compensate for the lack of functional protein in NNG3. This may lead to an up-regulation in the synthesis of the mRNA transcript in an attempt to produce functional type I PKC protein.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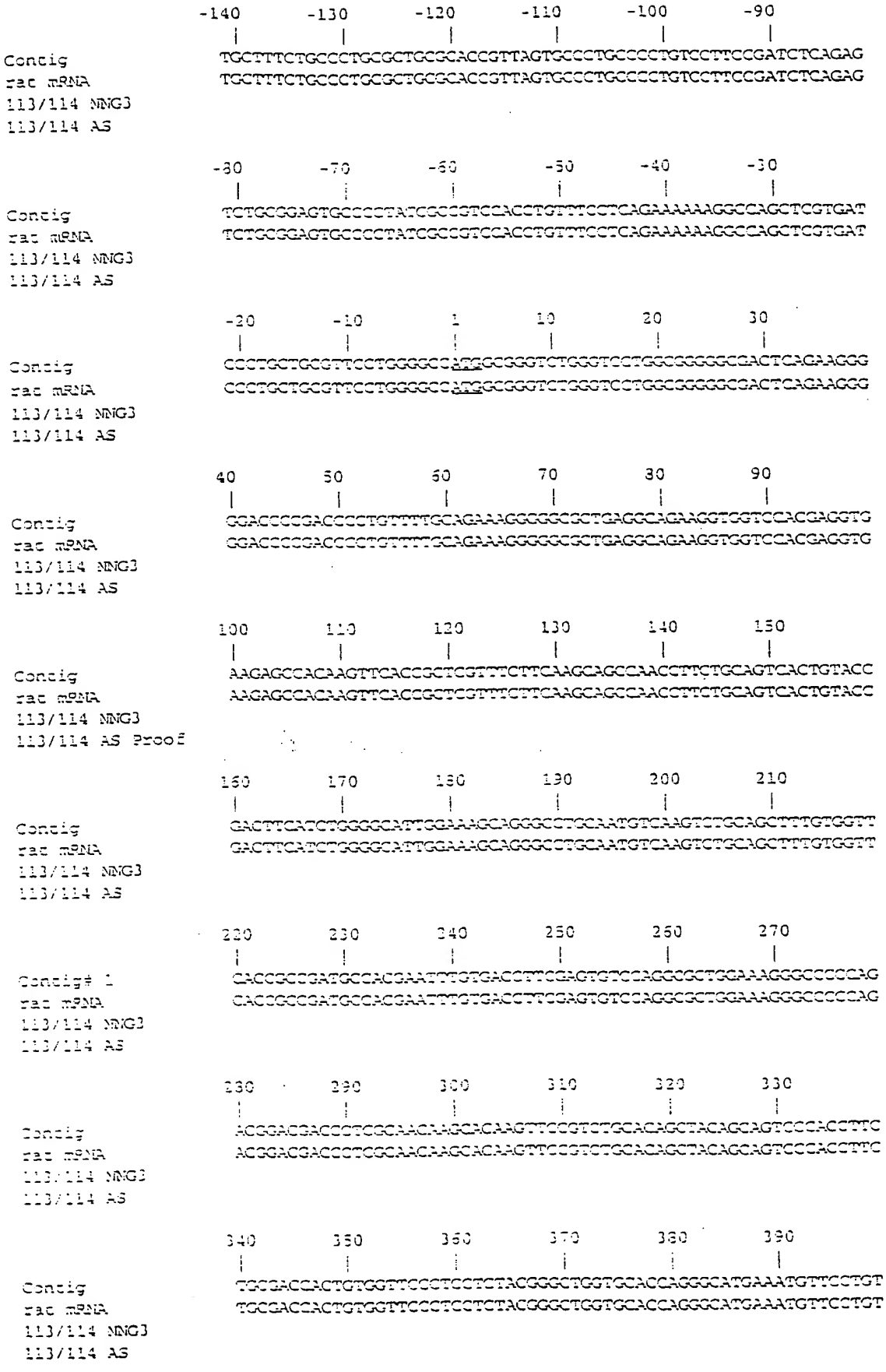
F344
DA
D1Mit1
5.5 cM ±1.1
NNG3/
R158
20.25 cM ±2
D1Mgh7
R191
4.5 cM
±0.5
NNG3/
R158
6.2 cM
±0.56
GU10
NNG3/R158 /
R93
5.5 cM
± 1.6
D1Mgh5

FIGURE 1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113/114 AS
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-80 -70 -60 -50 -40 -30
Contig
rat mRNA
113/114 NNG3
113/114 AS
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-20 -10 1 10 20 30
Contig
rat mRNA
113/114 NNG3
113/114 AS
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40 50 60 70 80 90
Contig
rat mRNA
113/114 NNG3
113/114 AS
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GGACCCCGACCCCTGTTTTGCAGAAAGGGGGCGCTGAGGCAGAAGGTGGTCCACGAGGTG
100 110 120 130 140 150
Contig
rat mRNA
113/114 NNG3
113/114 AS Proof
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AAGAGCCACAAGTTCACCGCTCGTTTCTTCAAGCAGCCAACCTTCTGCAGTCACTGTACC
160 170 180 190 200 210
Contig
rat mRNA
113/114 NNG3
113/114 AS
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GACTTCATCTGGGGCATTGGAAAGCAGGGCCTGCAATGTCAAGTCTGCAGCTTTGTGGTT
220 230 240 250 260 270
Contig# 1
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113/114 NNG3
113/114 AS
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CACCGCCGATGCCACGAATTTGTGACCTTCGAGTGTCCAGGCGCTGGAAAGGGCCCCCAG
280 290 300 310 320 330
Contig
rat mRNA
113/114 NNG3
113/114 AS
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ACGGACGACCCTCGCAACAAGCACAAGTTCCGTGTGCACAGCTACAGCAGTCCCACCTTC
340 350 360 370 380 390
Contig
rat mRNA
113/114 NNG3
113/114 AS
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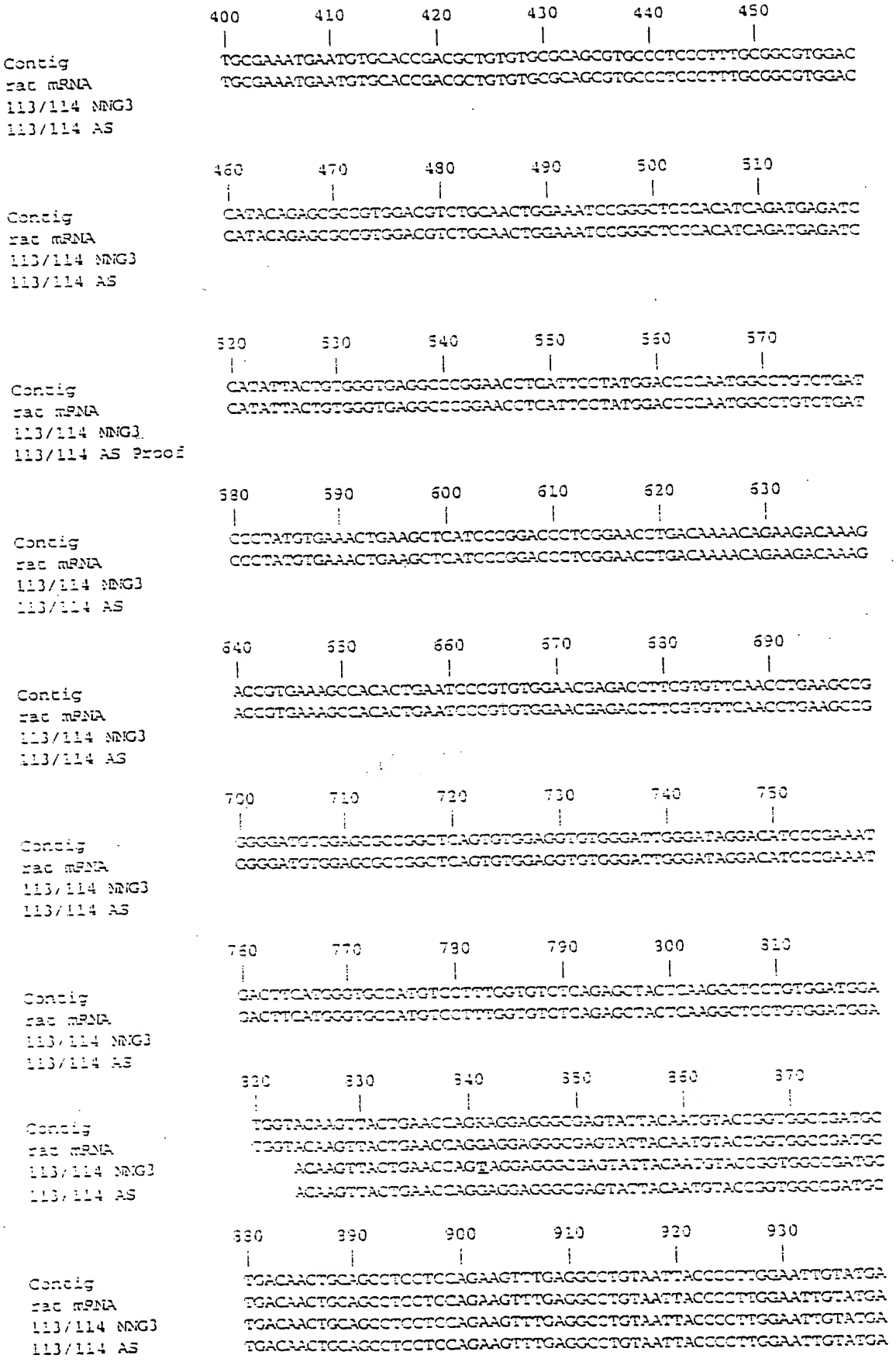
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113/114 NNG3
113/114 AS
460 470 480 490 500 510
Contig CATACAGAGCGCCGTGGACGTCTGCAACTGGAAATCCGGGCTCCCACATCAGATGAGATC
rat mRNA CATACAGAGCGCCGTGGACGTCTGCAACTGGAAATCCGGGCTCCCACATCAGATGAGATC
113/114 NNG3
113/114 AS
520 530 540 550 560 570
Contig CATATTACTGTGGGTGAGGCCCGGAACCTCATTCCTATGGACCCCAATGGCCTGTCTGAT
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113/114 NNG3
113/114 AS Proof
580 590 600 610 620 630
Contig CCCTATGTGAAACTGAAGCTCATCCCGGACCCTCGGAACCTGACAAAACAGAAGACAAAG
rat mRNA CCCTATGTGAAACTGAAGCTCATCCCGGACCCTCGGAACCTGACAAAACAGAAGACAAAG
113/114 NNG3
113/114 AS
640 650 660 670 680 690
Contig ACCGTGAAAGCCACACTGAATCCCGTGTGGAACGAGACCTTCGTGTTCAACCTGAAGCCG
rat mRNA ACCGTGAAAGCCACACTGAATCCCGTGTGGAACGAGACCTTCGTGTTCAACCTGAAGCCG
113/114 NNG3
113/114 AS
700 710 720 730 740 750
Contig GGGGATGTGGAGCGCCGGCTCAGTGTGGAGGTGTGGGATTGGGATAGGACATCCCGAAAT
rat mRNA GGGGATGTGGAGCGCCGGCTCAGTGTGGAGGTGTGGGATTGGGATAGGACATCCCGAAAT
113/114 NNG3
113/114 AS
760 770 780 790 800 810
Contig GACTTCATGGGTGCCATGTCCTTTGGTGTCTCAGAGCTACTCAAGGCTCCTGTGGATGGA
rat mRNA GACTTCATGGGTGCCATGTCCTTTGGTGTCTCAGAGCTACTCAAGGCTCCTGTGGATGGA
113/114 NNG3
113/114 AS
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Contig TGGTACAAGTTACTGAACCAGKAGGAGGGCGAGTATTACAATGTACCGGTGGCCGATGC
rat mRNA TGGTACAAGTTACTGAACCAGGAGGAGGGCGAGTATTACAATGTACCGGTGGCCGATGC
113/114 NNG3 ACAAGTTACTGAACCAGTAGGAGGGCGAGTATTACAATGTACCGGTGGCCGATGC
113/114 AS ACAAGTTACTGAACCAGGAGGAGGGCGAGTATTACAATGTACCGGTGGCCGATGC
880 890 900 910 920 930
Contig TGACAACTGCAGCCTCCTCCAGAAGTTTGAGGCCTGTAATTACCCCTTGGAATTGTATGA
rat mRNA TGACAACTGCAGCCTCCTCCAGAAGTTTGAGGCCTGTAATTACCCCTTGGAATTGTATGA
113/114 NNG3 TGACAACTGCAGCCTCCTCCAGAAGTTTGAGGCCTGTAATTACCCCTTGGAATTGTATGA
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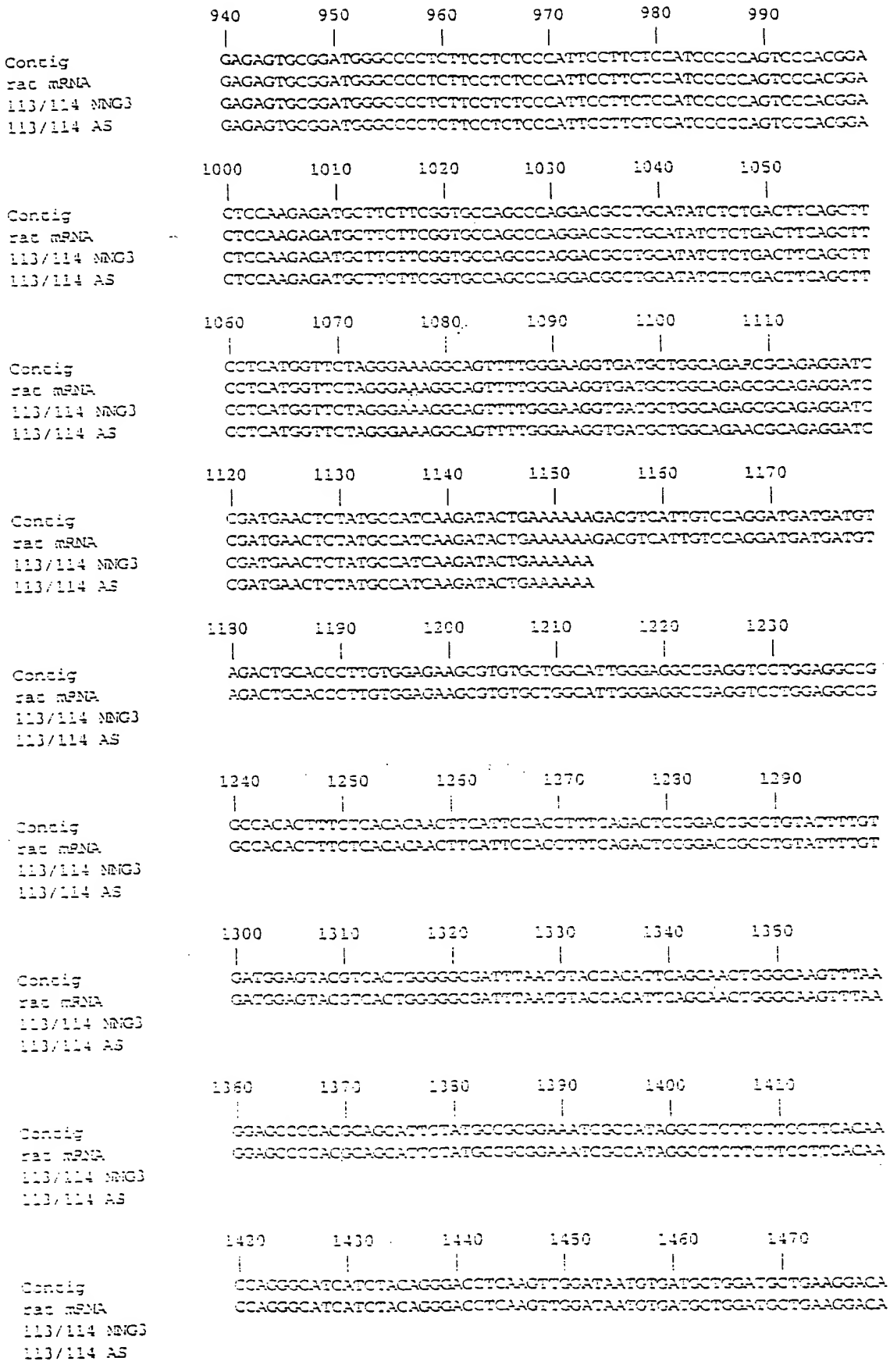
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Contig GAGAGTGCGGATGGGCCCCTCTTCCTCTCCCATTCCTTCTCCATCCCCCAGTCCCACGGA
rat mRNA GAGAGTGCGGATGGGCCCCTCTTCCTCTCCCATTCCTTCTCCATCCCCCAGTCCCACGGA
113/114 NNG3 GAGAGTGCGGATGGGCCCCTCTTCCTCTCCCATTCCTTCTCCATCCCCCAGTCCCACGGA
113/114 AS GAGAGTGCGGATGGGCCCCTCTTCCTCTCCCATTCCTTCTCCATCCCCCAGTCCCACGGA
1000 1010 1020 1030 1040 1050
Contig CTCCAAGAGATGCTTCTTCGGTGCCAGCCCAGGACGCCTGCATATCTCTGACTTCAGCTT
rat mRNA CTCCAAGAGATGCTTCTTCGGTGCCAGCCCAGGACGCCTGCATATCTCTGACTTCAGCTT
113/114 NNG3 CTCCAAGAGATGCTTCTTCGGTGCCAGCCCAGGACGCCTGCATATCTCTGACTTCAGCTT
113/114 AS CTCCAAGAGATGCTTCTTCGGTGCCAGCCCAGGACGCCTGCATATCTCTGACTTCAGCTT
1060 1070 1080 1090 1100 1110
Contig CCTCATGGTTCTAGGGAAAGGCAGTTTTGGGAAGGTGATGCTGGCAGAGCGCAGAGGATC
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113/114 NNG3 CCTCATGGTTCTAGGGAAAGGCAGTTTTGGGAAGGTGATGCTGGCAGAGCGCAGAGGATC
113/114 AS CCTCATGGTTCTAGGGAAAGGCAGTTTTGGGAAGGTGATGCTGGCAGAACGCAGAGGATC
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113/114 AS CGATGAACTCTATGCCATCAAGATACTGAAAAAA
1180 1190 1200 1210 1220 1230
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113/114 NNG3
113/114 AS
1240 1250 1260 1270 1280 1290
Contig GCCACACTTTCTCACACAACTTCATTCCACCTTTCAGACTCCGGACCGCCTGTATTTTGT
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113/114 NNG3
113/114 AS
1300 1310 1320 1330 1340 1350
Contig GATGGAGTACGTCACTGGGGGCGATTTAATGTACCACATTCAGCAACTGGGCAAGTTTAA
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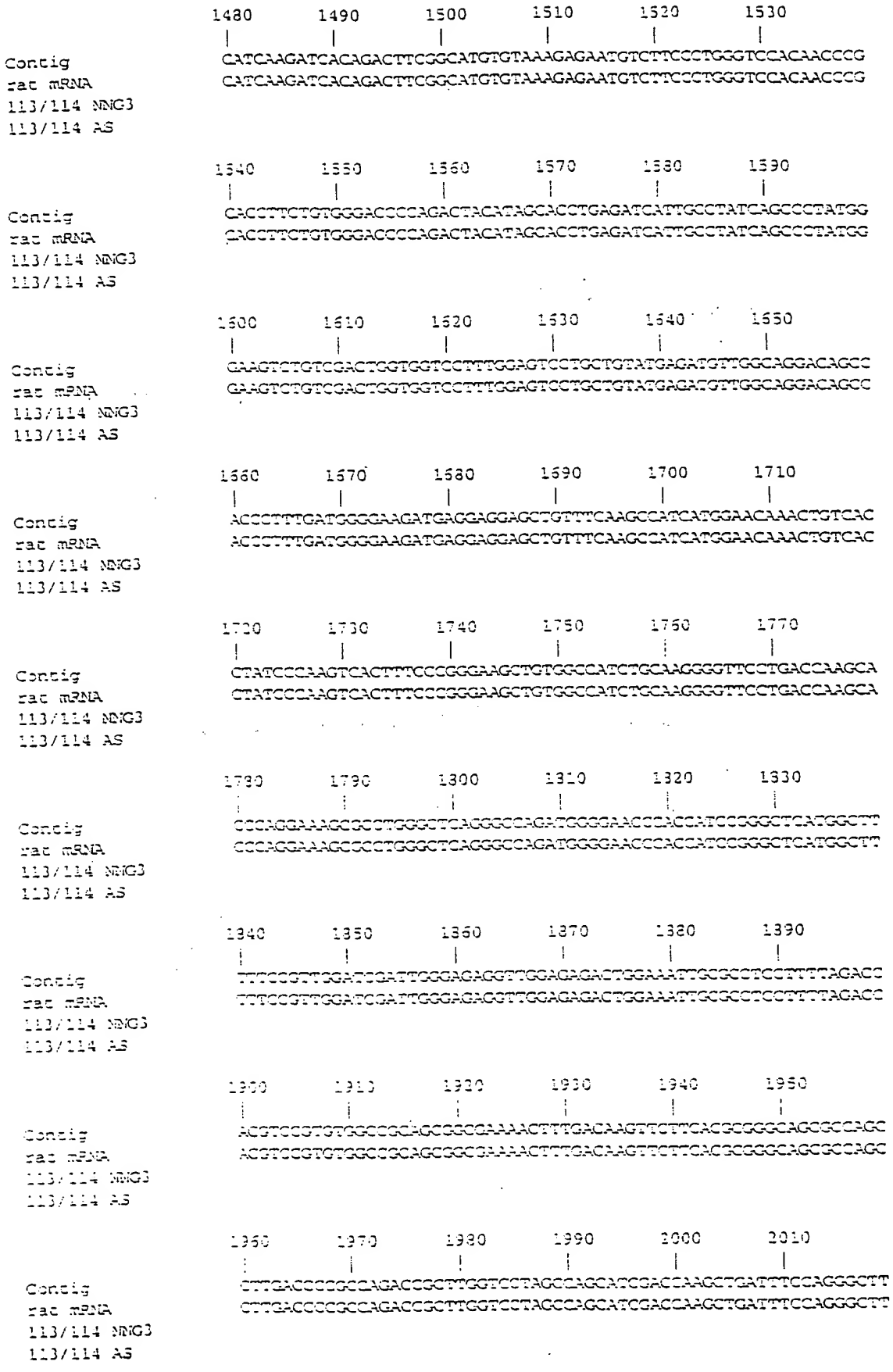
113/114 NNG3
113/114 AS
1360 1370 1380 1390 1400 1410
Contig GGAGCCCCACGCAGCATTCTATGCCGCGGAAATCGCCATAGGCCTCTTCTTCCTTCACAA
rat mRNA GGAGCCCCACGCAGCATTCTATGCCGCGGAAATCGCCATAGGCCTCTTCTTCCTTCACAA
113/114 NNG3
113/114 AS
1420 1430 1440 1450 1460 1470
Contig CCACGGCATCATCTACAGGGACCTCAAGTTGGATAATGTGATGCTGGATGCTGAAGGACA
rat mRNA CCACGGCATCATCTACAGGGACCTCAAGTTGGATAATGTGATGCTGGATGCTGAAGGACA
113/114 NNG3
113/114 AS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Contig
rat mRNA
113/114 NNG3
113/114 AS
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CATCAAGATCACAGACTTCGGCATGTGTAAAGAGAATGTCTTCCCTGGGTCCACAACCCG
1540 1550 1560 1570 1580 1590
Contig
rat mRNA
113/114 NNG3
113/114 AS
CACCTTCTGTGGGACCCCAGACTACATAGCACCTGAGATCATTGCCTATCAGCCCTATGG
CACCTTCTGTGGGACCCCAGACTACATAGCACCTGAGATCATTGCCTATCAGCCCTATGG
1600 1610 1620 1630 1640 1650
Contig
rat mRNA
113/114 NNG3
113/114 AS
GAAGTCTGTCGACTGGTGGTCCTTTGGAGTCCTGCTGTATGAGATGTTGGCAGGACAGCC
GAAGTCTGTCGACTGGTGGTCCTTTGGAGTCCTGCTGTATGAGATGTTGGCAGGACAGCC
1660 1670 1680 1690 1700 1710
Contig
rat mRNA
113/114 NNG3
113/114 AS
ACCCTTTGATGGGGAAGATGAGGAGGAGCTGTTTCAAGCCATCATGGAACAAACTGTCAC
ACCCTTTGATGGGGAAGATGAGGAGGAGCTGTTTCAAGCCATCATGGAACAAACTGTCAC
1720 1730 1740 1750 1760 1770
Contig
rat mRNA
113/114 NNG3
113/114 AS
CTATCCCAAGTCACTTTCCCGGGAAGCTGTGGCCATCTGCAAGGGGTTCCTGACCAAGCA
CTATCCCAAGTCACTTTCCCGGGAAGCTGTGGCCATCTGCAAGGGGTTCCTGACCAAGCA
1780 1790 1800 1810 1820 1830
Contig
rat mRNA
113/114 NNG3
113/114 AS
CCCAGGAAAGCGCCTGGGCTCAGGGCCAGATGGGGAACCCACCATCCGGGCTCATGGCTT
CCCAGGAAAGCGCCTGGGCTCAGGGCCAGATGGGGAACCCACCATCCGGGCTCATGGCTT
1840 1850 1860 1870 1880 1890
Contig
rat mRNA
113/114 NNG3
113/114 AS
TTTCCGTTGGATCGATTGGGAGAGGTTGGAGAGACTGGAAATTGCGCCTCCTTTTAGACC
TTTCCGTTGGATCGATTGGGAGAGGTTGGAGAGACTGGAAATTGCGCCTCCTTTTAGACC
1900 1910 1920 1930 1940 1950
Contig
rat mRNA
113/114 NNG3
113/114 AS
ACGTCCGTGTGGCCGCAGCGGCGAAAACTTTGACAAGTTCTTCACGCGGGCAGCGCCAGC
ACGTCCGTGTGGCCGCAGCGGCGAAAACTTTGACAAGTTCTTCACGCGGGCAGCGCCAGC
1960 1970 1980 1990 2000 2010
Contig
rat mRNA
113/114 NNG3
113/114 AS
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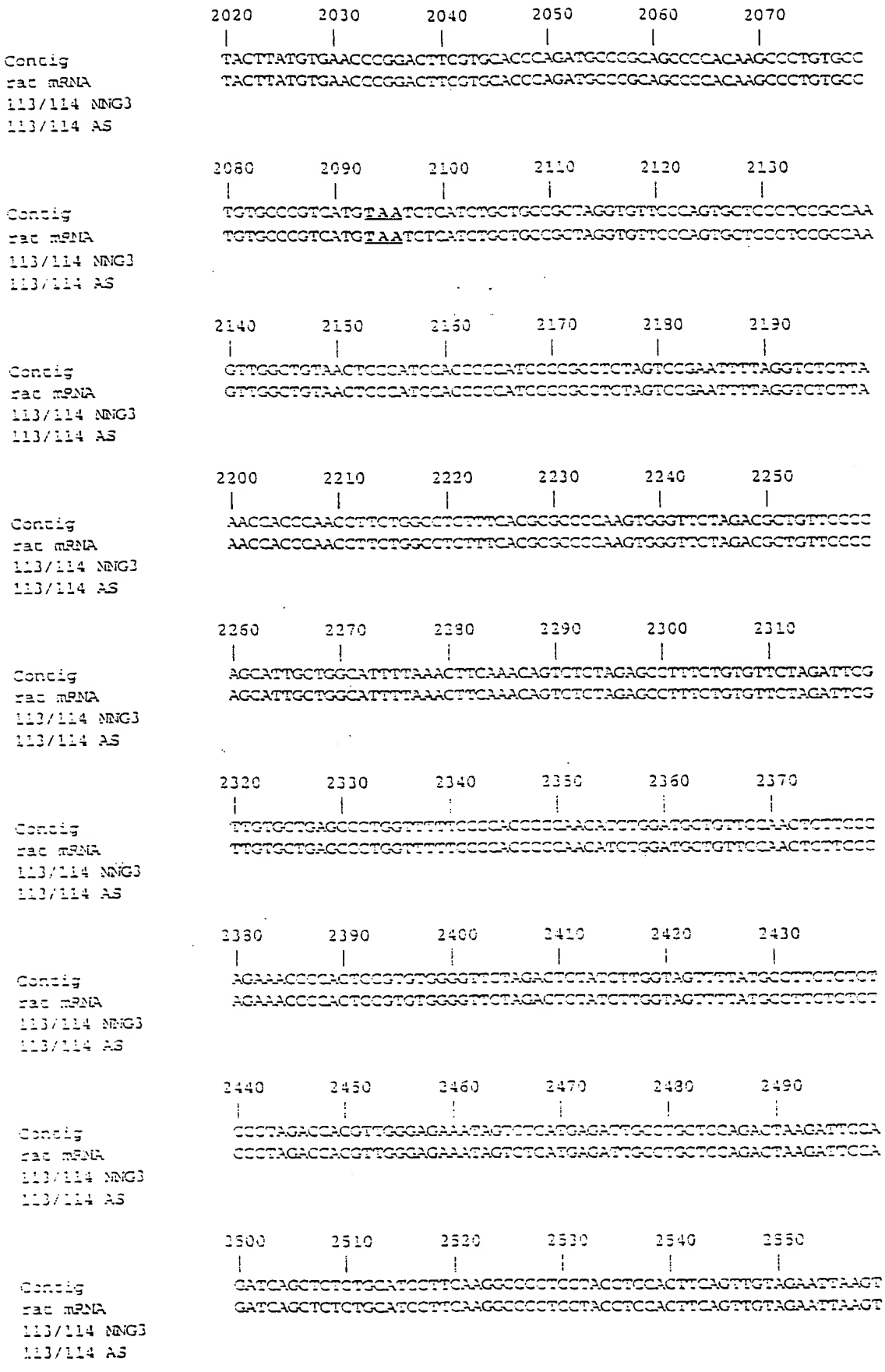
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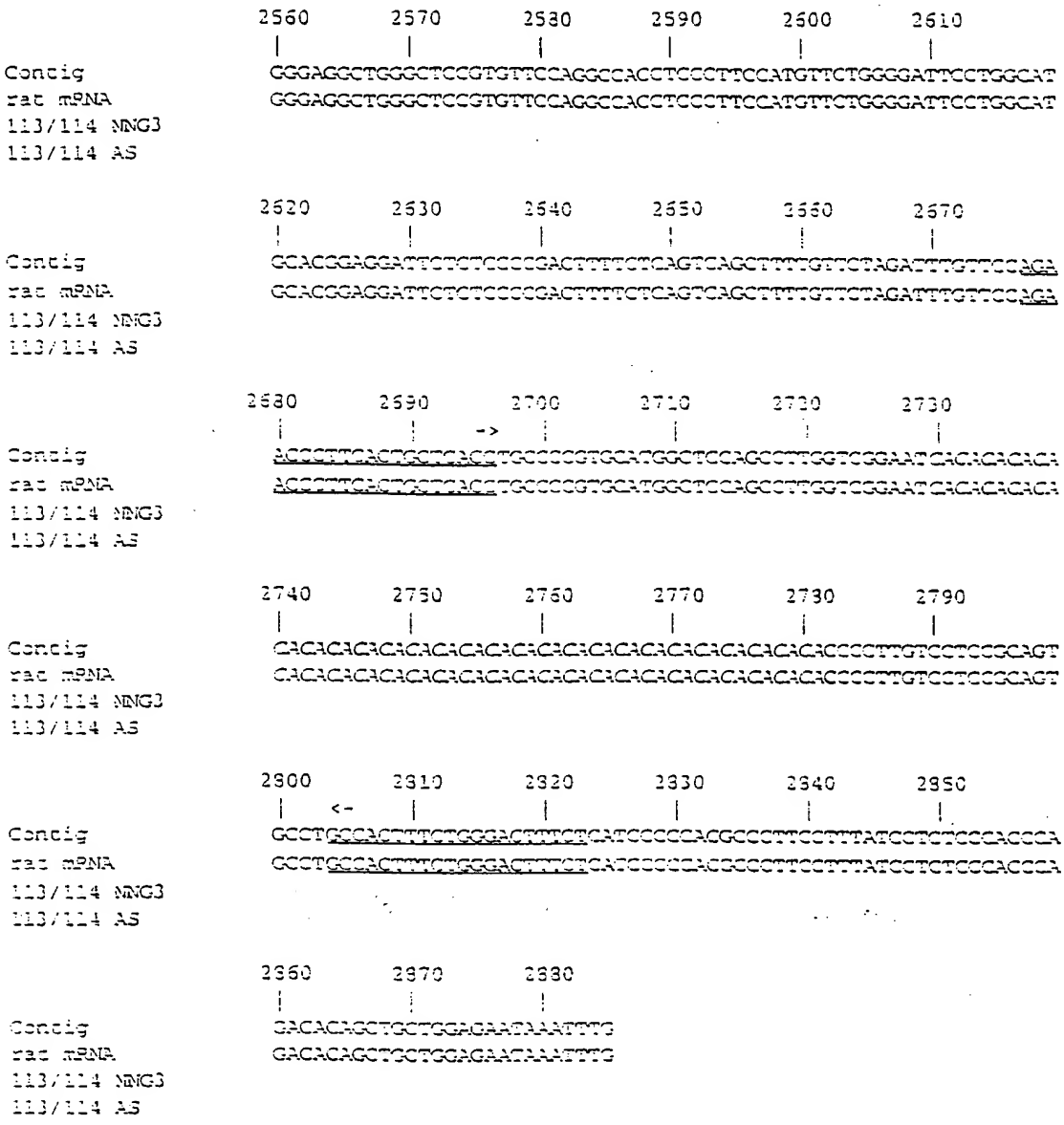
Contig TACTTATGTGAACCCGGACTTCGTGCACCCAGATGCCCGCAGCCCCACAAGCCCTGTGCC
rat mRNA TACTTATGTGAACCCGGACTTCGTGCACCCAGATGCCCGCAGCCCCACAAGCCCTGTGCC
113/114 NNG3
113/114 AS
2080 2090 2100 2110 2120 2130
Contig TGTGCCCGTCATGTAATCTCATCTGCTGCCGCTAGGTGTTCCCAGTGCTCCCTCCGCCAA
rat mRNA TGTGCCCGTCATGTAATCTCATCTGCTGCCGCTAGGTGTTCCCAGTGCTCCCTCCGCCAA
113/114 NNG3
113/114 AS
2140 2150 2160 2170 2180 2190
Contig GTTGGCTGTAACTCCCATCCACCCCCATCCCCCGCCTCTAGTCCGAATTTTAGGTCTCTTA
rat mRNA GTTGGCTGTAACTCCCATCCACCCCCATCCCCCGCCTCTAGTCCGAATTTTAGGTCTCTTA
113/114 NNG3
113/114 AS
2200 2210 2220 2230 2240 2250
Contig AACCACCCAACCTTCTGGCCTCTTTCACGCGCCCCAAGTGGGTTCTAGACGCTGTTCCCC
rat mRNA AACCACCCAACCTTCTGGCCTCTTTCACGCGCCCCAAGTGGGTTCTAGACGCTGTTCCCC
113/114 NNG3
113/114 AS
2260 2270 2280 2290 2300 2310
Contig AGCATTGCTGGCATTTTAAACTTCAAACAGTCTCTAGAGCCTTTCTGTGTTCTAGATTCG
rat mRNA AGCATTGCTGGCATTTTAAACTTCAAACAGTCTCTAGAGCCTTTCTGTGTTCTAGATTCG
113/114 NNG3
113/114 AS
2320 2330 2340 2350 2360 2370
Contig TTGTGCTGAGCCCTGGTTTTTCCCCACCCCCAACATCTGGATGCTGTTCCAACTCTTCCC
rat mRNA TTGTGCTGAGCCCTGGTTTTTCCCCACCCCCAACATCTGGATGCTGTTCCAACTCTTCCC
113/114 NNG3
113/114 AS
2380 2390 2400 2410 2420 2430
Contig AGAAACCCCACTCCGTGTGGGGTTCTAGACTCTATCTTGGTAGTTTTATGCCTTCTCTCT
rat mRNA AGAAACCCCACTCCGTGTGGGGTTCTAGACTCTATCTTGGTAGTTTTATGCCTTCTCTCT
113/114 NNG3
113/114 AS
2440 2450 2460 2470 2480 2490
Contig CCCTAGACCACGTTGGGAGAAATAGTCTCATGAGATTGCCTGCTCCAGACTAAGATTCCA
rat mRNA CCCTAGACCACGTTGGGAGAAATAGTCTCATGAGATTGCCTGCTCCAGACTAAGATTCCA
113/114 NNG3
113/114 AS
2500 2510 2520 2530 2540 2550
Contig GATCAGCTCTCTGCATCCTTCAAGGCCCCTCCTACCTCCACTTCAGTTGTAGAATTAAGT
rat mRNA GATCAGCTCTCTGCATCCTTCAAGGCCCCTCCTACCTCCACTTCAGTTGTAGAATTAAGT
113/114 NNG3
113/114 AS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rat mRNA GGGAGGCTGGGCTCCGTGTTCCAGGCCACCTCCCTTCCATGTTCTGGGGATTCCTGGCAT
113/114 MNG3
113/114 AS
2620 2630 2640 2650 2660 2670
Contig GCACGGAGGATTCTCTCCCCGACTTTTCTCAGTCAGCTTTTGTTCTAGATTTGTTCCAGA
rat mRNA GCACGGAGGATTCTCTCCCCGACTTTTCTCAGTCAGCTTTTGTTCTAGATTTGTTCCAGA
113/114 MNG3
113/114 AS
2680 2690 -> 2700 2710 2720 2730
Contig ACCCTTCACTGCTCACCTGCCCCCGTGCATGGCTCCAGCCTTGGTCGGAATCACACACACA
rat mRNA ACCCTTCACTGCTCACCTGCCCCCGTGCATGGCTCCAGCCTTGGTCGGAATCACACACACA
113/114 MNG3
113/114 AS
2740 2750 2760 2770 2780 2790
Contig CACACACACACACACACACACACACACACACACACACACACACACCCCTTGTCCTCCGCAGT
rat mRNA CACACACACACACACACACACACACACACACACACACACACACACCCCTTGTCCTCCGCAGT
113/114 MNG3
113/114 AS
2800 <- 2810 2820 2830 2840 2850
Contig GCCTGCCACTTTCTGGGACTTTCTCATCCCCCACGCCCTTCCTTTATCCTCTCCCACCCA
rat mRNA GCCTGCCACTTTCTGGGACTTTCTCATCCCCCACGCCCTTCCTTTATCCTCTCCCACCCA
113/114 MNG3
113/114 AS
2860 2870 2880
Contig GACACAGCTGCTGGAGAATAAATTTG
rat mRNA GACACAGCTGCTGGAGAATAAATTTG
113/114 MNG3
113/114 AS

FIGURE 2

```
-141                                                                  TGCTTTC
-134 TGCCCTGCGCTGCGCACCGTTAGTGCCCTGCCCCTGTCCTTCCGATCTCAGAGTCTGCGGAGTGCCC
 -67 CTATCGCCGTCCACCTGTTTCCTCAGAAAAAAGGCCAGCTCGTGATCCCTGCTGCGTTCCTGGGGCC

  Start Ala Gly Leu Gly Pro Gly Gly Gly Asp Ser Glu Gly Gly Pro Arg Pro     17
  1  ATG GCG GGT CTG GGT CCT GGC GGG GGC GAC TCA GAA GGG GGA CCC CGA CCC

    Leu Phe Cys Arg Lys Gly Ala Leu Arg Gln Lys Val Val His Glu Val Lys     34
 52 CTG TTT TGC AGA AAG GGG GCG CTG AGG CAG AAG GTG GTC CAC GAG GTG AAG

    Ser His Lys Phe Thr Ala Arg Phe Phe Lys Gln Pro Thr Phe Cys Ser His     51
103 AGC CAC AAG TTC ACC GCT CGT TTC TTC AAG CAG CCA ACC TTC TGC AGT CAC

    Cys Thr Asp Phe Ile Trp Gly Ile Gly Lys Gln Gly Leu Gln Cys Gln Val     68
154 TGT ACC GAC TTC ATC TGG GGC ATT GGA AAG CAG GGC CTG CAA TGT CAA GTC

    Cys Ser Phe Val Val His Arg Arg Cys His Glu Phe Val Thr Phe Glu Cys     85
205 TGC AGC TTT GTG GTT CAC CGC CGA TGC CAC GAA TTT GTG ACC TTC GAG TGT

    Pro Gly Ala Gly Lys Gly Pro Gln Thr Asp Asp Pro Arg Asn Lys His Lys    102
256 CCA GGC GCT GGA AAG GGC CCC CAG ACG GAC GAC CCT CGC AAC AAG CAC AAG

    Phe Arg Leu His Ser Tyr Ser Ser Pro Thr Phe Cys Asp His Cys Gly Ser    119
307 TTC CGT CTG CAC AGC TAC AGC AGT CCC ACC TTC TGC GAC CAC TGT GGT TCC

    Leu Leu Tyr Gly Leu Val His Gln Gly Met Lys Cys Ser Cys Cys Glu Met    136
358 CTC CTC TAC GGG CTG GTG CAC CAG GGC ATG AAA TGT TCC TGT TGC GAA ATG

    Asn Val His Arg Arg Cys Val Arg Ser Val Pro Ser Leu Cys Gly Val Asp    153
409 AAT GTG CAC CGA CGC TGT GTG CGC AGC GTG CCC TCC CTT TGC GGC GTG GAC

    His Thr Glu Arg Arg Gly Arg Leu Gln Leu Glu Ile Arg Ala Pro Thr Ser    170
460 CAT ACA GAG CGC CGT GGA CGT CTG CAA CTG GAA ATC CGG GCT CCC ACA TCA

    Asp Glu Ile His Ile Thr Val Gly Glu Ala Arg Asn Leu Ile Pro Met Asp    187
511 GAT GAG ATC CAT ATT ACT GTG GGT GAG GCC CGG AAC CTC ATT CCT ATG GAC

    Pro Asn Gly Leu Ser Asp Pro Tyr Val Lys Leu Lys Leu Ile Pro Asp Pro    204
562 CCC AAT GGC CTG TCT GAT CCC TAT GTG AAA CTG AAG CTC ATC CCG GAC CCT

    Arg Asn Leu Thr Lys Gln Lys Thr Lys Thr Val Lys Ala Thr Leu Asn Pro    221
613 CGG AAC CTG ACA AAA CAG AAG ACA AAG ACC GTG AAA GCC ACA CTG AAT CCC

    Val Trp Asn Glu Thr Phe Val Phe Asn Leu Lys Pro Gly Asp Val Glu Arg    238
664 GTG TGG AAC GAG ACC TTC GTG TTC AAC CTG AAG CCG GGG GAT GTG GAG CGC

    Arg Leu Ser Val Glu Val Trp Asp Trp Asp Arg Thr Ser Arg Asn Asp Phe    255
715 CGG CTC AGT GTG GAG GTG TGG GAT TGG GAT AGG ACA TCC CGA AAT GAC TTC

    Met Gly Ala Met Ser Phe Gly Val Ser Glu Leu Leu Lys Ala Pro Val Asp    272
766 ATG GGT GCC ATG TCC TTT GGT GTC TCA GAG CTA CTC AAG GCT CCT GTG GAT

                                Mutant NNG3
                                   STOP
    Gly Trp Tyr Lys Leu Leu Asn Gln Glu Glu Gly Glu Tyr Tyr Asn Val Pro    289
817 GGA TGG TAC AAG TTA CTG AAC CAG GAG GAG GGC GAG TAT TAC AAT GTA CCG
                                   TAG

    Val Ala Asp Ala Asp Asn Cys Ser Leu Leu Gln Lys Phe Glu Ala Cys Asn    306
868 GTG GCC GAT GCT GAC AAC TGC AGC CTC CTC CAG AAG TTT GAG GCC TGT AAT
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    Tyr Pro Leu Glu Leu Tyr Glu Arg Val Arg Met Gly Pro Ser Ser Ser Pro    323
919 TAC CCC TTG GAA TTG TAT GAG AGA GTG CGG ATG GGC CCC TCT TCC TCT CCC

    Ile Pro Ser Pro Ser Pro Ser Pro Thr Asp Ser Lys Arg Cys Phe Phe Gly    340
970 ATT CCT TCT CCA TCC CCC AGT CCC ACG GAC TCC AAG AGA TGC TTC TTC GGT


    Ala Ser Pro Gly Arg Leu His Ile Ser Asp Phe Ser Phe Leu Met Val Leu    357
1021 GCC AGC CCA GGA CGC CTG CAT ATC TCT GAC TTC AGC TTC CTC ATG GTT CTA

    Gly Lys Gly Ser Phe Gly Lys Val Met Leu Ala Glu Arg Arg Gly Ser Asp    374
1072 GGG AAA GGC AGT TTT GGG AAG GTG ATG CTG GCA GAG CGC AGA GGA TCC GAT

    Glu Leu Tyr Ala Ile Lys Ile Leu Lys Lys Asp Val Ile Val Gln Asp Asp    391
1123 GAA CTC TAT GCC ATC AAG ATA CTG AAA AAA GAC GTC ATT GTC CAG GAT GAT

    Asp Val Asp Cys Thr Leu Val Glu Lys Arg Val Leu Ala Leu Gly Gly Arg    408
1174 GAT GTA GAC TGC ACC CTT GTG GAG AAG CGT GTG CTG GCA TTG GGA GGC CGA

    Gly Pro Gly Gly Arg Pro His Phe Leu Thr Gln Leu His Ser Thr Phe Gln    425
1225 GGT CCT GGA GGC CGG CCA CAC TTT CTC ACA CAA CTT CAT TCC ACC TTT CAG

    Thr Pro Asp Arg Leu Tyr Phe Val Met Glu Tyr Val Thr Gly Gly Asp Leu    442
1276 ACT CCG GAC CGC CTG TAT TTT GTG ATG GAG TAC GTC ACT GGG GGC GAT TTA

    Met Tyr His Ile Gln Gln Leu Gly Lys Phe Lys Glu Pro His Ala Ala Phe    459
1327 ATG TAC CAC ATT CAG CAA CTG GGC AAG TTT AAG GAG CCC CAC GCA GCA TTC

    Tyr Ala Ala Glu Ile Ala Ile Gly Leu Phe Phe Leu His Asn Gln Gly Ile    476
1378 TAT GCC GCG GAA ATC GCC ATA GGC CTC TTC TTC CTT CAC AAC CAG GGC ATC

    Ile Tyr Arg Asp Leu Lys Leu Asp Asn Val Met Leu Asp Ala Glu Gly His    493
1429 ATC TAC AGG GAC CTC AAG TTG GAT AAT GTG ATG CTG GAT GCT GAA GGA CAC

    Ile Lys Ile Thr Asp Phe Gly Met Cys Lys Glu Asn Val Phe Pro Gly Ser    510
1480 ATC AAG ATC ACA GAC TTC GGC ATG TGT AAA GAG AAT GTC TTC CCT GGG TCC

    Thr Thr Arg Thr Phe Cys Gly Thr Pro Asp Tyr Ile Ala Pro Glu Ile Ile    527
1531 ACA ACC CGC ACC TTC TGT GGG ACC CCA GAC TAC ATA GCA CCT GAG ATC ATT

    Ala Tyr Gln Pro Tyr Gly Lys Ser Val Asp Trp Trp Ser Phe Gly Val Leu    544
1582 GCC TAT CAG CCC TAT GGG AAG TCT GTC GAC TGG TGG TCC TTT GGA GTC CTG

    Leu Tyr Glu Met Leu Ala Gly Gln Pro Pro Phe Asp Gly Glu Asp Glu Glu    561
1633 CTG TAT GAG ATG TTG GCA GGA CAG CCA CCC TTT GAT GGG GAA GAT GAG GAG

    Glu Leu Phe Gln Ala Ile Met Glu Gln Thr Val Thr Tyr Pro Lys Ser Leu    578
1684 GAG CTG TTT CAA GCC ATC ATG GAA CAA ACT GTC ACC TAT CCC AAG TCA CTT

    Ser Arg Glu Ala Val Ala Ile Cys Lys Gly Phe Leu Thr Lys His Pro Gly    595
1735 TCC CGG GAA GCT GTG GCC ATC TGC AAG GGG TTC CTG ACC AAG CAC CCA GGA

    Lys Arg Leu Gly Ser Gly Pro Asp Gly Glu Pro Thr Ile Arg Ala His Gly    612
1786 AAG CGC CTG GGC TCA GGG CCA GAT GGG GAA CCC ACC ATC CGG GCT CAT GGC

    Phe Phe Arg Trp Ile Asp Trp Glu Arg Leu Glu Arg Leu Glu Ile Ala Pro    629
1837 TTT TTC CGT TGG ATC GAT TGG GAG ACG TTG GAG AGA CTG GAA ATT GCG CCT

    Pro Phe Arg Pro Arg Pro Cys Gly Arg Ser Gly Glu Asn Phe Asp Lys Phe    646
1888 CCT TTT AGA CCA CGT CCG TGT GGC CGC AGC GGC GAA AAC TTT GAC AAG TTC

    Phe Thr Arg Ala Ala Pro Ala Leu Thr Pro Pro Asp Arg Leu Val Leu Ala    663
1939 TTC ACG CGG GCA GCG CCA GCC TTG ACC CCG CCA GAC CGC TTG GTC CTA GCC
```

```
     Ser Ile Asp Gln Ala Asp Phe Gln Gly Phe Thr Tyr Val Asn Pro Asp Phe    680
1990 AGC ATC GAC CAA GCT GAT TTC CAG GGC TTT ACT TAT GTG AAC CCG GAC TTC

     Val His Pro Asp Ala Arg Ser Pro Thr Ser Pro Val Pro Val Pro Val Met    697
2041 GTG CAC CCA GAT GCC CGC AGC CCC ACA AGC CCT GTG CCT GTG CCC GTC ATG
     Stop                                                                  697
2092 TAA TCTCATCTGCTGCCGCTAGGTGTTCCCAGTGCTCCCTCCGCCAAGTTGGCTGTAACTCCCATC
2158 CACCCCCATCCCCCGCCTCTAGTCCGAATTTTAGGTCTCTTAAACCACCCAACCTTCTGGCCTCTTTC
2225 ACGCGCCCCAAGTGGGTTCTAGACGCTGTTCCCCAGCATTGCTGGCATTTTAAACTTCAAACAGTCT
2292 CTAGAGCCTTTCTGTGTTCTAGATTCGTTGTGCTGAGCCCTGGTTTTTCCCCACCCCCAACATCTGG
2359 ATGCTGTTCCAACTCTTCCCAGAAACCCCACTCCGTGTGGGGTTCTAGACTCTATCTTGGTAGTTTT
2426 ATGCCTTCTCTCTCCCTAGACCACGTTGGGAGAAATAGTCTCATGAGATTGCCTGCTCCAGACTAAG
2493 ATTCCAGATCAGCTCTCTGCATCCTTCAAGGCCCCTCCTACCTCCACTTCAGTTGTAGAATTAAGTG
2560 GGAGGCTGGGCTCCGTGTTCCAGGCCACCTCCCTTCCATGTTCTGGGGATTCCTGGCATGCACGGAG
2627 GATTCTCTCCCCGACTTTTCTCAGTCAGCTTTTGTTCTAGATTTGTTCCAGAACCCTTCACTGCTCA
2694 CCTGCCCCGTGCATGGCTCCAGCCTTGGTCGGAATCACACACACACACACACACACACACACACACA
2761 CACACACACACACACACACACCCCTTGTCCTCCGCAGTGCCTGCCACTTTCTGGGACTTTCTCATCC
2828 CCCACGCCCTTCCTTTATCCTCTCCCACCCAGACACAGCTGCTGGAGAATAAATTTG
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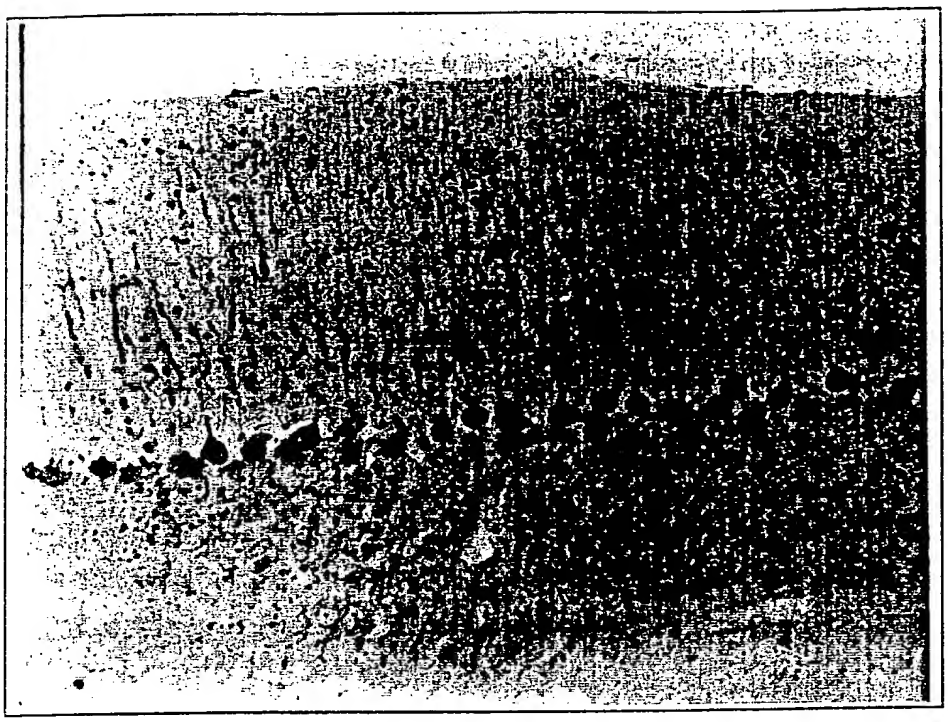
FIGURE 3

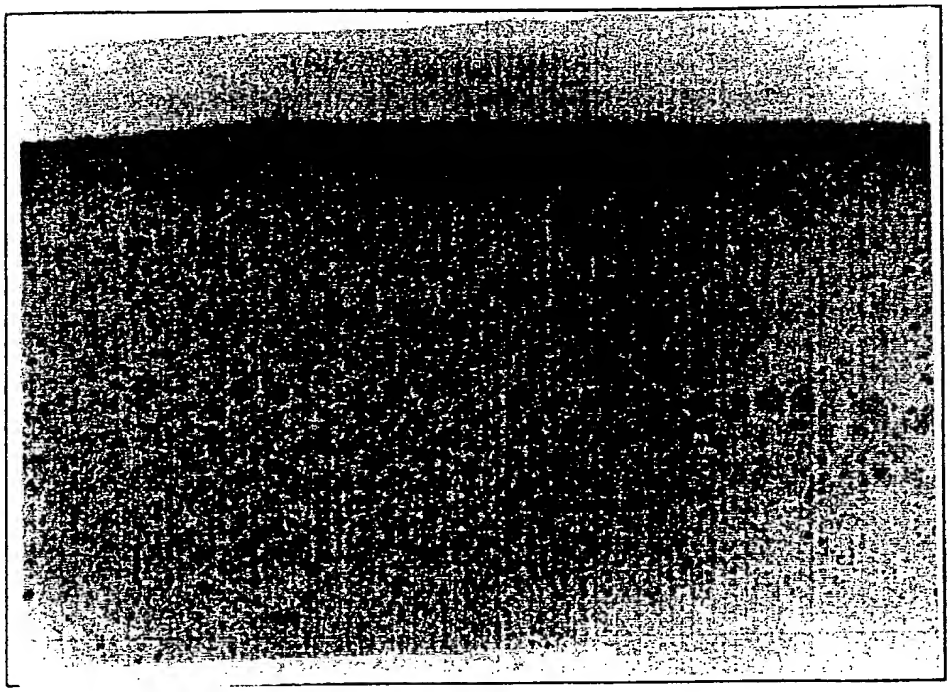
A



Granule cell layer

Purkinje cell layer

B



Granule cell layer

Purkinje cell layer

FIGURE 4

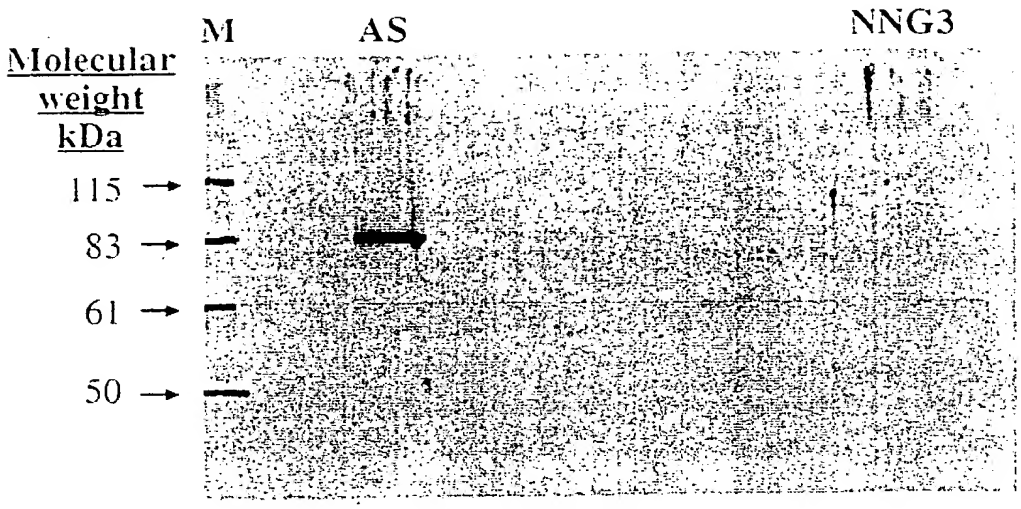


FIGURE 5

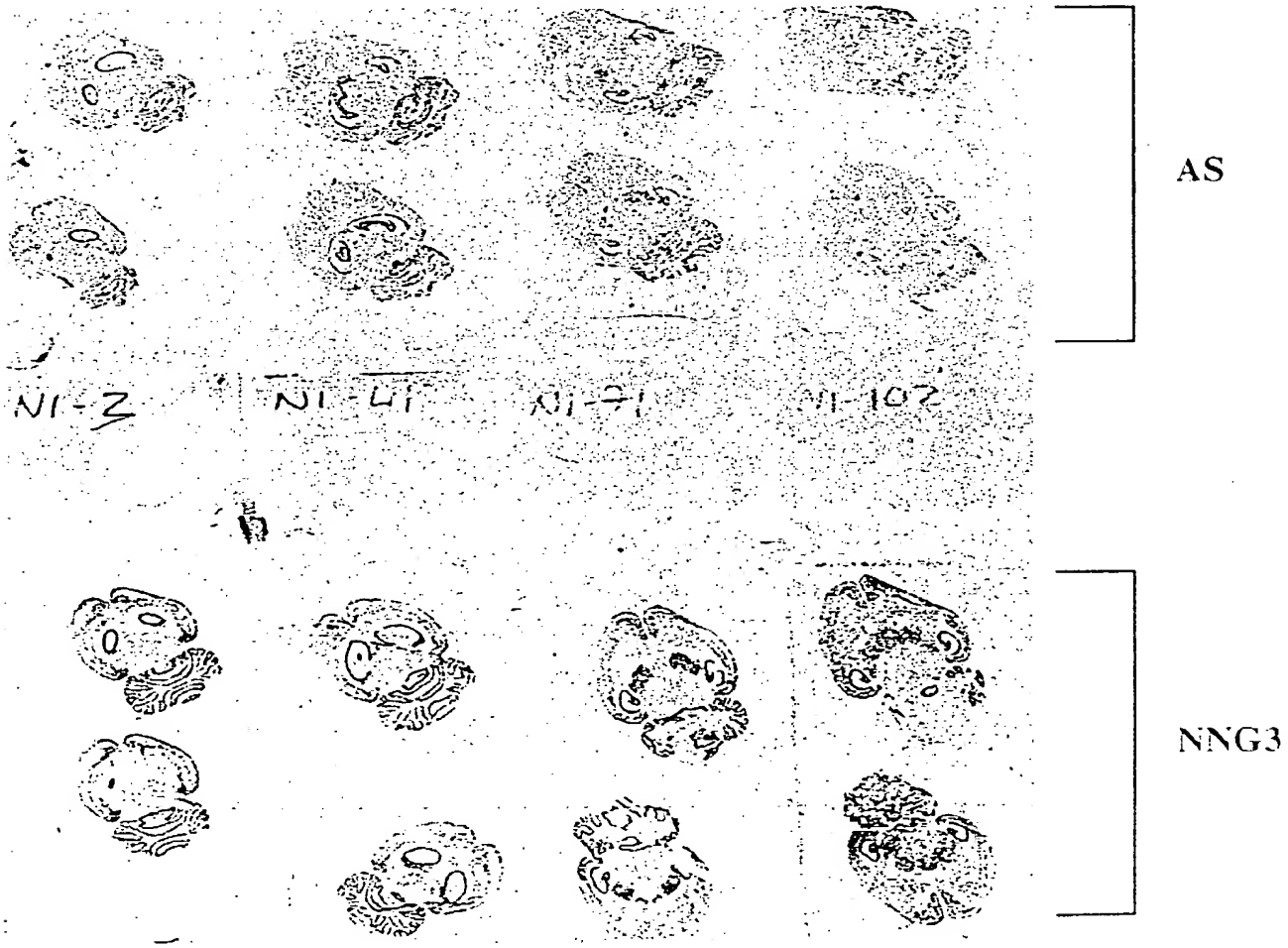


FIGURE 6

PTO 08/00000
Cruikshank - Fairmeadow
15-3-00